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Enzymatic Production of Biodiesel from Fats Extracted From Lamb Meat Using Supercritical Co2

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A thesis
Submitted to
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ABSTRACT

Due to the reduction in fossil fuel reserves and associated environmental impacts from using petroleum fuels, biodiesel has been presented as a feasible alternative because it sustainable and environmentally friendly. In this study it is purposed to investigate the feasibility of enzymatic production of biodiesel from waste animal fats using supercritical fluid technology for the extraction and reaction. The operating conditions that resulted in the optimum extraction and biodiesel production yield were identified. To the best knowledge of the investigators, the waste animal fat extracted by supercritical CO_2 (SC-CO_2) for the production of biodiesel in SC-CO_2 media has never been investigated.

Waste fat extracted from lamb meat was selected as feedstock for the transesterification reaction using Novozym 435 as a biocatalyst for biodiesel production and SC-CO_2 as an extraction solvent and reaction medium. Fatty acid methyl ester (FAME) analysis was accomplished using gas chromatography equipped with ionization detector (GC-FID). The effect of temperature in the range of 35-55 °C, pressure in the range of 300-500 bar and SC-CO_2 flow rate in the range of 3-5 ml min^{-1} on the fat extraction effectiveness and yield were investigated and optimized using response surface methodology. To evaluate the feasibility of using Supercritical fluid extraction (SFE) as an alternative extraction method, its fat extraction yield was compared to that using soxhlet extraction. Statistical analysis was done using Minitab 15 software. In addition, the effects of temperature in the range of 35-60°C, methanol molar ratio in the range of 3:1-6:1 and enzyme loading in the range of 10-50%, on reaction rate and yield were also tested. The experimental results were used to fit a suitable reaction kinetic model to estimate the model parameters using non-linear regression analysis.

The results indicated that effective SFE requires dry meat sample at moderate temperature. At optimum conditions, the system was capable of extracting up to 87.4% of the total fat content from freeze dried and grind meat sample at 45 °C, 500 bars and 3 ml min^{-1}. The statistical analysis indicated that the yield was a function of extraction temperature and SC-CO_2 flow rate, whereas that extraction pressure showed insignificant effect.

Biodiesel production by enzymatic transesterification of extracted lamb meat fat with methanol, using Novozym 435 proved to be of high potential, with a conversion of almost 40%. Effect of reaction conditions and reaction kinetics were investigated. The optimal conditions for transesterification of lamb meat using Novozym 435 in SC-CO_2 reaction medium were: 50 °C, 30% loading, 4:1 methanol to fat molar ratio and 25 hr reaction. When subjected to repeated uses, Novozym 435 showed significant loss in its activity. The experimental results were used to fit simplified model based on Ping Pong Bi Bi with methanol inhibition to determine kinetic parameters using non-linear regression technique.

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SYMBOLS AND ABBREVIATIONS

Abbreviation	
[M]	Methanol concentration, g g^{-1}
[S]	Substrate concentration, g g^{-1}
ANOVA	Analysis of Variance
BF_3	Boron trifluoride
CO	Carbon monoxide
CO_2	Carbon dioxide
CLA	Conjugate Linoleic Acid
DG	Diglycerides
DW	Distilled water
F	Flow rate, ml min^{-1}
FA	Fatty Acid
FAAE	Fatty Acid Alkyl Ester
FAME	Fatty acids methyl ester
FFA	Free Fatty Acid
FFD	Full Factorial Design
FID	Flame Ionization detector
GC	Gas Chromatography
GC-FID	Gas Chromatography with Flame Ionization detector
GHG	Green house gas
H_2	Hydrogen
HLFM	Healthy Low Fat Meat

HPLC	High Performance Liquid Chromatography
hr	hour
i	component i
IMF	Intramuscular Fat
Imm.	Immobilized Enzyme
min	minute
MG	Monoglycerides
MUFA	Monounsaturated Fatty Acid
K_F	Fat apparent Michaelis–Menten constant, g g^{-1}
K_{IM}	Methanol apparent inhibition constant, g g^{-1}
K_{IS}	Substrate apparent inhibition constant, m^3mol^{-1}
K_m	Michaelis–Menten constant, mol m^3
K_M	Methanol apparent Michaelis–Menten constant, g g^{-1}
K_{S1}	Substrate (1) apparent Michaelis–Menten constant, mol m^3
K_{S2}	Substrate (2) apparent Michaelis–Menten constant, mol m^3
N_2	Nitrogen
N_2O	Nitrous oxide
NO_x	Nitrogen oxides
P	Pressure, bar
PLU	Propyl Laurate Unit
PM	Particular Matter
RSM	Response Surface Methodology
PUFA	Polyunsaturated Fatty Acid
SC-CO_2	Supercritical carbon dioxide
SCA	Supercritical alcohols

SCF	Supercritical fluid
SCM	Supercritical Methanol
SFE	Supercritical fluid Extraction
SFA	Saturated Fatty Acids
T	Temperature, °C
TG	Triglycerides
THF	Tetrahydrofuran
UHP	Ultra High purity
UFA	Unsaturated fatty acid

Greek symbols

β_0	Regression Constant
β_i	Linear coefficient
β_{ii}	Quadratic coefficient
β_{ij}	interaction coefficient
v	Initial reaction rate, g g^{-1} hr^{-1}
v_{max}	Initial Maximum reaction rate, g g^{-1} hr^{-1}

CHAPTER ONE: INTRODUCTION

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1.1 MOTIVATION

Continuous exploration and consumption of fossil fuels have led to decline in worldwide oil reserves. As the world energy demand is increasing, the most sufficient way to meet the growing demand is by finding alternative fuels. From the point of environment protection, finding alternative fuels that are sustainable and environment friendly is essential.

More than a century ago, Rudolf Diesel tested the ability of using vegetable oils as fuel for his engine [1, 2]. In the 1930s and 1940s, vegetable oils were used as diesel fuel for emergency situations. At that time, vegetable oil fuels were not competitive because they were more expensive than petroleum fuels, and therefore the idea was abandoned. With the worries about petroleum fuel availability and latest increases in petroleum prices, using vegetable oils back in diesel engines has regained attention.

A number of studies have shown that triglycerides hold promise as alternative diesel engine fuels [1, 3]. This has an advantage of being available, renewable with higher cetane number and biodegradability [4]. However, the main disadvantage of oils is their high viscosity and low volatility [1, 5]. Therefore, direct use of triglycerides is generally unacceptable and not practical. In addition, it has many problems including; coking, carbon depositing and gelling of the lubricating oil [6]. To overcome direct use problems, dilution, pyrolysis, cracking and transesterification methods have been suggested [7, 8]. Among all these methods, transesterification has been used widely as a favourable method. Transesterification reaction of triglycerides, mostly called alcoholysis, is an important reaction that produces fatty acids alkyl esters (FAAE) [6, 9]. It was reported that using biodiesel results in a reduction of unburned hydrocarbons, carbon monoxide (CO) and particular matter (PM) formation [10, 11].

Several methods of transesterification using alkalis [12-16] and acids [14, 17-21] as catalysts and enzymatic transesterification using lipase in presence and absence of solvents have been reported [22-27]. Most of the commercial biodiesel processes requires

the use of a catalyst, either alkaline or acid catalyst, which requires a recovery unit to separate transesterification reaction products and remove the catalyst. These disadvantages of using catalyst could be eliminated by carrying out non catalytic reaction. Sake and Kusdiana [28] developed a method using supercritical methanol (SCM) where triglycerides fatty acids were converted to methyl esters without using any catalyst. Sake and Kusdiana [28] and Madras et al. [29] reported the advantage of using supercritical alcohols (SCA), especially methanol, over conventional catalyzed methods where a process requires short reaction time and no need for reaction product purification. But, this process is energy intensive at supercritical conditions of methanol.

To overcome drawbacks of the conventional catalysts and supercritical alcohol methods, more sustainable method has been suggested. Enzymatic biodiesel approach showed promising results due to their high selectivity and mild operative conditions. Enzymatic transesterification reaction is similar to conventional transesterification, except that they are catalyzed by a variety of biological catalysts rather than chemical catalysts. In contrast to conventional processes, biocatalysts can transesterify triglycerides with high free fatty acids (FFA) contents [30]. Lipases are biological catalysts that represent a category of enzymes that synthesize lipids and fats. Lipase catalyzed transesterification of triglyceride (TG) has been investigated by several investigators [30-34]. One common drawback with the use of enzyme-based processes is the high cost of the enzyme compared to conventional chemical catalysts; therefore, their recycle is required. This is possible through enzyme immobilization.

Immobilization of enzymes has generally been used to attain reusable enzyme with lower production cost [23]. Thus, immobilized form of lipase has been used in most of transesterification processes [23, 33, and 35]. Besides enzyme reusability, main advantage of using immobilized lipase as a catalyst is enhanced activity and stability [36, 37].

Several researches have been carried out to produce biodiesel in solvent systems. Presently, industries are facing problems in using conventional solvents due to environmental worries. In the last couple of decades, enzyme catalyzed reactions in supercritical carbon dioxide (SC-CO_2) has been studied. Previously, most of the studies were concerning on proving ability of using biocatalyst in SC-CO_2 whereas recent studies are focusing on obtaining good yield and conversions.

Vegetable oils consist of triglyceride of straight chains of fatty acids. With the high cost of biodiesel associated from vegetable oils expensive cost, researchers are looking for low cost feedstocks that can overcome this high cost problem. For that waste oils, cooking and frying oils and fats from animal sources were proposed. The main drawback that faced the use of animal fats their solid state and high melting points, therefore, it was suggested to carry out the transesterification reaction in organic solvents to dissolve the fat. However, organic solvent use is not recommended since this require a recovery unit and it residue in the meat after fat extraction. To overcome this, supercritical fluids (SCFs) are introduced.

During the past decades, SCFs have been investigated as alternative solvents for reactions rather than using conventional solvents. Among all supercritical fluids, SC-CO_2 is the most secure choice as a consequence of its availability. In general, CO_2 is non-toxic, non-flammable, environmentally friendly and recyclable fluid [38]. Thus, reactions in SC-CO_2 media become the preferable route for synthesis chemical synthesis.

On the other hand, people are always looking for healthier low fat food, such as red lean meats. Mostly, meat contains fats that present at the surface and intramuscular fats (IMF). Meat processors trim meat outer surface to obtain this low fat product. However, this does not remove IMF, therefore, a need for an extraction solvent is required. Previously, researchers used conventional solvents to removed undesirable fats from meat. Nevertheless, conventional solvents can't be used with food samples, since there will be some solvent residue remaining in the sample. For that SC-CO_2 has been suggested to take the place as a non-toxic and non-hazardous extraction solvent. Many researchers have used SC-CO_2 to remove fat from meat products. It has been used to extract fat from beef patties, fish muscle and cattle brain. Furthermore, SC-CO_2 is more capable of removing intramuscular fat.

1.2 THESIS OBJECTIVES

The main objective of this study was to investigate the biodiesel synthesis with lipase (Novozym 435) as catalyst from waste animal fat using SC-CO_2 as a reaction medium and extraction solvent. It was proposed to use the fat extracted from lamb meat

as a feed stock and convert it to fatty acid methyl ester through transesterification reaction in the presence of Novozym 435.

In biodiesel production, effect of reaction time and temperature, the molar ratio, enzyme loading and enzyme reusability was investigated. Besides these, enzyme kinetic was studied. In supercritical extraction, the effects of samples pre-treatment, extraction temperature pressure and flow rates on reaction yield and system efficiency and process optimization were examined.

1.3 THESIS ORGANIZATION

A brief summary of the organization of the thesis is shown in this section. There are five chapters besides the introduction chapter. Chapter one, this chapter, starts with the recent fuel demand that requires finding alternative fuels, mainly biodiesel. It also gives an overview of the biodiesel production which leads to the development of the transesterification process. Research objectives are also stated in this chapter. Chapter two is a literature review where a study of the previous research works done on biodiesel synthesis by different methods, lipases, reaction systems and possible feedstock have been reviewed. In addition, an overview on the applications of supercritical fluids is also provided. Material and methods used in this research are summarized in chapter three. This includes chemicals and enzyme, used method in sample preparation, fat extraction, biodiesel production as well as full factorial experimental design and applied statistical analysis. In chapter four, most important obtained results are shown and discussed. In chapter four, influence of different pre-treatment methods are presented, followed by an optimization of supercritical extraction conditions. This is followed by showing effect of molar ratio, reaction time and temperature, enzyme loading and reusability on reaction yield. At the end of the chapter products chemical analysis and enzyme kinetic study are conducted. Finally, conclusion and scope of future works are presented in chapter five.

CHAPTER TWO: LITERATURE REVIEW

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This chapter summarizes methods using of biodiesel as an alternative fuel, lipase as a biocatalyst and supercritical carbon dioxide as an extraction solvent and a reaction medium. It is arranged in the following manner: In the first section, biodiesel as an alternative fuel, its properties, uses, production techniques are presented, with special focus on enzymatic transesterification and possible feedstocks. In the second section, supercritical fluids, their properties and applications are discussed; the third section discusses relevant previous works related to the enzymatic reactions in supercritical carbon dioxide as a reaction medium. Finally, a literature on meat is presented; where meat and fat compositions are discussed with examples of previous works on supercritical carbon dioxide extraction of fat from meat matrix are mentioned.

2.1 BIODIESEL

Nowadays, most of the world energy needs are supplied through fossil sources such as petrol, natural gas and coal. These sources are limited and will be eventually consumed; therefore, the search for alternatives is essential. According to an estimate, the reserves will last for another 218 years for coal, 41 years for oil and 63 years for natural gas [2]. Alternatives to petroleum diesel should have similar chemical and physical properties. In addition, they should be technically feasible, economically competitive and environmentally friendly [39, 40].

Biodiesel has arisen as a possible alternative for petroleum diesel as results of the similarities that biodiesel has with petroleum-diesel [41]. This section presents literature on using biodiesel as an alternative fuel and on its production technologies.

2.1.1 Biodiesel properties and uses

Biodiesel fuel has many advantages over petroleum fuel. Some of the most touted include the fact that biodiesel is non-toxic, biodegradable, renewable and do not

contribute to net accumulation of the greenhouse gas (GHG) [42, 43]. As well as have lower sulphur and aromatic content, higher cetane number and flash point [5, 7, and 10]. Other benefits of biodiesel include increased lubricity and lower emissions of certain harmful exhaust gases in comparison to petroleum diesel fuel [44].

Comparing petroleum diesel fuel to biodiesel, Schumacher et al. [45] reported that the biodiesel results in a 45.2% reduction in total hydrocarbon emissions, 47.5% reduction in CO emissions and 66.7% reduction in PM emissions whereas, Demirbas [44] reported a 42.7% reduction in CO and 55.3% in PM emissions relatively to standard diesel fuel. These effects are generally attributed to the higher cetane number and oxygen content of biodiesel fuel.

Although the biodiesel environmental considerations are positive, one negative exhaust characteristic noted from biodiesel is an increase in nitrogen oxides (NO_x) emissions. Schumacher et al. [11] mentioned that neat biodiesel has 11.5% increase in NO_x emissions over petroleum diesel. Demirbas [44] reported that 100% biodiesel has 13.2% increase in NO_x emission relative to the standard diesel fuel. However, reports show that actual reductions in NO_x emissions are possible with some modifications in combustion temperatures and injection timing [46].

Transportation sector is one of the fossil fuels consumers and in the same time the biggest contributor to environmental pollution. Thus, biodiesel is recommended to be used is diesel engine cars, buses, trucks, construction equipment, boats, generators and oil home heating units [42, 47].

2.1.2 Biodiesel relevance

Biodiesel is a name given to diesel like fuels produced from renewable feedstocks. Renewable source refers to raw materials that can be obtained by the growth of plants or production of livestock. The biodiesel can be either used in its pure form or by mixing it with conventional diesel fuel [5, 41, and 48].

Biodiesel consists of the methyl esters of the fatty acids contained in the vegetable oils and animal fats (consist mainly of TG). A part from vegetable oils and animal fats, biodiesel can also be produced from waste oil, greases and algae. Direct use of vegetable

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oil has several negative aspects. Main drawbacks are their high viscosity and low volatility. These lead to have incomplete combustion in diesel engines, therefore, carbon deposition [7, 12, 49, and 50]. However, the direct use of vegetable oils as biodiesel is possible by mixing them with conventional diesel in an appropriate ratio, but this mixing will be impractical for a long term uses in the engine [39, 51, and 52]. Therefore, considerable efforts have been made to develop vegetable oil derivatives that have properties near those of the petroleum-based diesel fuels.

Pyrolysis (cracking), microemulsion and transesterification are the possible methods to reduce direct feedstock use problems [6-8]. The first two methods are costly and yield to have low quality biodiesel whereas the latter, transesterification, is the most common method to transform oil into biodiesel, which is the focus of this study.

2.1.3 Transesterification

Transesterification is the common method used to transform TG into biodiesel. This consists of the reaction between TG and an acyl-acceptor [53]. Carboxylic acids, alcohols or another ester can be used as acyl-acceptor. This produce glycerol when alcohol is used as acyl-acceptor or triacylglycerol when ester is used as by products [50, 54-57]. Transesterification process using the catalyst is called catalytic transesterification process, whereas that without catalyst is called non-catalytic transesterification process [6, 40, 48, and 58]. Moreover, catalytic process is divided into two types; homogenous and heterogeneous process depending of the catalyst used.

Transesterification is a chemical process of transforming large and branched TG into smaller and straight chain molecules, which is similar in size to the molecules of the species present in diesel fuel [59, 60]. Stoichiometrically, for each mole of TG three moles of alcohol are required. But in general, a higher molar ratio is mostly used in order to achieve maximum biodiesel production. This molar ratio value depends on the type of used feedstock, type of catalyst, temperature and reaction time. Methanol, ethanol and propanol are the most commonly used alcohols. In fact, biodiesel yield is independent of the type of the alcohol used but the alcohol selection depends on the cost [50]. It is different than esterification; in transesterification, ester bonds are broken first then followed by hydroxyl bond, whereas in esterification hydroxyl bonds are broken before

ester bonds resulting to have a glycerol as by-product in transesterification and water in esterification. The typical form of the reaction is illustrated in Figure 2.1 where R_1, R_2 and R_3 represent the fatty acid chains [61].

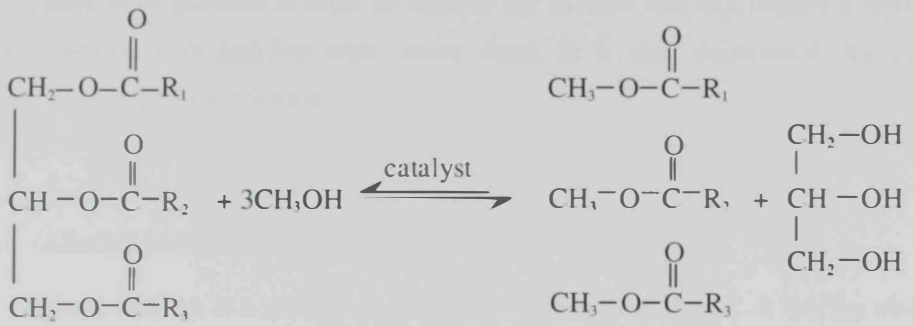

Figure 2.1: Transesterification of triglycerides to fatty acid esters

Transesterification can be carried in a number of ways by using different catalytic processes. For example, it can be carried out using an alkali catalyst, acid catalyst and biocatalyst or using alcohols in their supercritical state [62, 63] as shown in Figure 2.2. Overall transesterification is a sequence of three reactions; TG is first converted to a diacylglycerol (DG) and one fatty acid ester, then the DG is converted to monoacylglycerol (MG) giving an additional fatty acid ester, and finally the MG is converted to glycerol giving the last fatty acid ester.

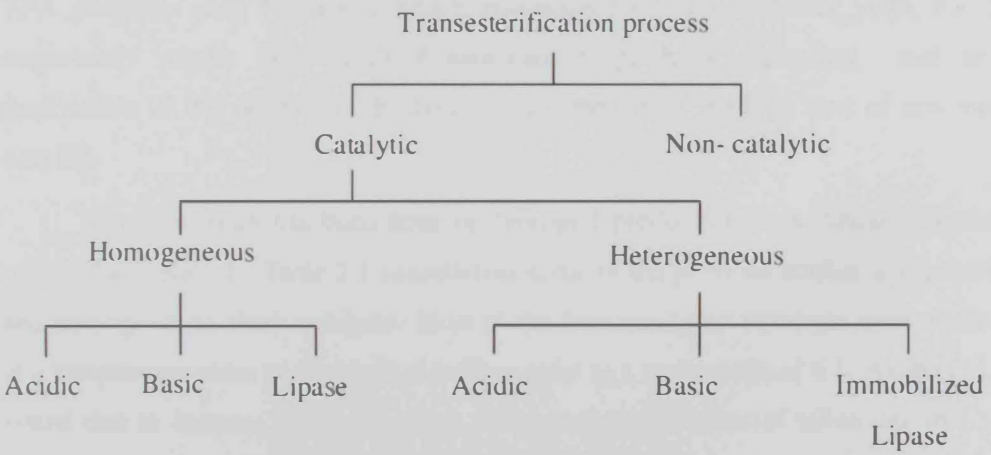

Figure 2.2: Summary of transesterification methods

Catalyst promotes hydrolysis of the TGs to produce fatty acids and glycerol, with the last being a by-products. By the end of the transesterification, produced biodiesel and glycerol have to be purified in order to remove the catalyst and will require a separation step by washing with distilled water many times. It is well understood that catalyst selection is an important criterion.

2.1.3.1 Alkaline catalyst transesterification

A base catalyst is a chemical with a pH value greater than 7. It has the ability to give extra electrons. Sodium hydroxide (NaOH), potassium hydroxide (KOH) and sodium methoxide (CH_3ONa) are the most common homogeneous base catalysts employed during alkaline transesterification [8, 39, and 50]. The base catalyzed process is most commonly used because of its relative ease. It can be performed at low temperature and pressure and yield high conversion (98%) within a short time [7].

In spite of the high yield conversion that can be obtained, this method has some drawbacks. Most important limitation of the base catalysis method is the process sensitivity to both FFA and water contents. It works perfectly when the FFA and moisture contents are less than certain limits, usually below 0.5wt% for FFA, [64, 65]. In case TGs where FFA contents exceed this limit, pre-treatment step is required. The presence of FFA promotes soap formation, which consumes the catalyst, lower yield, and more importantly results in difficult downstream by-products separation and product purification [5, 6]. 60-90% of biodiesel cost comes from the high cost of raw material cost [5].

Great research has been done on biodiesel production from different feedstocks using alkali catalysts. Table 2.1 summarizes some of the previous studies using methanol and homogeneous alkali catalysts. Most of the base catalyzed reactions were carried out at a temperature close to the alcohol boiling point at a molar ratio of 6:1. Akoh et al. [66] stated that to increase biodiesel yield, a stoichiometric excess of substrates (6:1 molar ratio of methanol to oil) is favoured. In addition, alkali catalyst needs effluent treatment.

Kinetic studies on alkaline processes have been done by different researchers. In 1986, Freedman et al. [67] investigated a second order for the reverse reaction and pseudo-first order or second order on forward reaction, depending on reaction conditions. In 2000, Darnoko and Cheryan [68] studied KOH catalysis kinetic of palm oil. They concluded that the reaction is pseudo second-order at the initial stages followed by first-order or zero-order kinetics at later stages.

Table 2.1: Summary of previous researches on transesterification using homogeneous alklaine catalyzed process.

Catalyst	Fat/Oil	Alcohol	Temperature (°C)	Molar ratio (Methanol: Oil)	Reaction time	Yield (%)	Reference
NaOH	Duck tallow	Methanol	65	6:1	3 hr	62.3	[69]
	Treated waste cooking oil	Methanol	50	7:1	1 hr	88.9	[70]
	Sunflower oil	Methanol	65	6:1	--	86.7	[71]
	Neat canola oil	Methanol	70	6:1	15 min	93.5	[72]
	Treated used frying oil	Methanol	60	7:1	20 min	88.8	[72]
	Soybean oil	Methanol	45	6:1	20 min	100	[73]
	Sunflower oil	Methanol	60	6:1	1.5 hr	97.1	[74]
KOH	Duck tallow	Methanol	65	6:1	3 hr	79.7	[69]
	Karanja oil	Methanol	65	6:1	3 hr	97-98	[75]
	Pongamia pinnata	Methanol	45	10:1	1.5 hr	83	[76]
	Pongamia pinnata	Methanol	60	10:1	1.5 hr	92	[76]
	Sunflower oil	Methanol	65	6:1	--	91.6	[71]
CH_3NaO	Duck tallow	Methanol	65	6:1	3 hr	79.3	[69]
	Sunflower oil	Methanol	65	6:1	--	99.3	[71]

Homogeneous catalysts have been used industrially for biodiesel production where produced biodiesel and glycerol have to be purified to remove the catalyst. This purification process requires large quantities of water and energy to be accomplished. Thus, heterogeneous catalysts have been suggested to overcome drawback. Heterogeneous catalysts can be separated easily from the system at the end by filtration and could be reused [50, 77]. Alkaline earth oxides [78], zeolites [3], calcined hydrotalcites [16, 79] and Magnesium and Calcium oxides [15, 77] have been suggested as heterogeneous catalysts, and showed good results. However, the high cost of the purified feedstock remains the main problem facing the alkaline catalyzed process.

2.1.3.2 Acid catalyst transesterification

The base-catalyzed transesterification processes described in section 2.1.3.1 are suitable for feedstocks with low FFA content. High FFA content, >0.5wt%, results in downstream separation complexity. Therefore other methods of transesterification must be used [5, 80].

Acid catalysis is the second conventional way of producing biodiesel. The idea is to use the TGs and alcohol with an acid as a catalyst instead of a base. Most commonly used acids are strong acids like sulphuric acid, sulphonic acid, phosphoric and hydrochloric [7].

Acid-catalyzed transesterification processes are not commercial as the base catalyzed process, mainly because strong acids are corrosive. In addition, acid catalyzed processes are too slow. Several hours that may take more than one day may be required in order to achieve high conversion. It is 4000 times slower than the base catalyst process [5, 12, and 58]. Above that, it requires high alcohol to oil molar ratio and more amounts of catalyst. Akoh et al. [66] stated that a molar ratio of 30:1 in a range of 55-80°C with 0.5 to 1 mol% catalyst concentration is required to achieve 99% conversion in 50 hr. On the other hand, acid catalysed processes offer an important advantage of independent on feedstock FFA content. That is because feedstock FFA is not converted to soap using this kind of catalysts. Therefore, biodiesel can be produced from low cost feedstock [61].

As mentioned before high FFA content requires pre-treatment step when base catalyst is used. In fact, acid catalysis can perform this pre-treatment step. For that reason, an acid and methanol mixture is added to the TGs. When equilibrium is reached, methanol, water and the mixture is removed from the reaction vessel by centrifugation [80]. This is followed by adding a fresh methanol and base catalyst to the oil in order to base catalyze the transesterification reaction.

Great investigations were performed on the use of acid catalysts. Freedman et al. [67] studied soybean oil transesterification with methanol using H_2SO_4 as a catalyst. They concluded that in order to achieve 90% conversion from 1wt% of H_2SO_4 and at 65°C, 69 hr and 30:1 molar ratio of alcohol to soybean oil are required. Canakci and Gerpen [81] investigated the effects of several parameters, and found that biodiesel conversion can be increased by increasing alcohol to oil molar ratio, increasing reaction temperature and acid catalyst concentration. Also they reported that the optimum conditions for the reaction are 60°C, 3 wt % H_2SO_4, 6:1 molar ratio of methanol to oil and a reaction time of 48 hr. Biodiesel yield increased from 87.8 to 95.1% when the reaction time was increased from 48 to 96 hr. Additionally, Bhatti et al. [17] assessed the possibility of producing biodiesel from waste chicken and mutton tallow using acid and base catalysis. Results showed that acid catalysis resulted in higher yield than base catalysis, which is expected due to the use of untreated feedstock. Using acid catalysis, 99% and 93.2% yields were obtained from chicken and mutton tallow, respectively. On the other hand, using base catalysis, yield of 88.1 and 78.3% were obtained from chicken and mutton tallow, respectively. Zheng et al. [82] studied acid catalyzed transesterification reaction kinetic of waste frying oil. They found that at 70°C and methanol to oil ratio of 250:1, transesterification was a first order and a conversion of 99% was obtained. Wang et al. [83] used 2% ferric sulphate to carry out esterification reaction before transesterification. Conversion of 97% of waste cooking oil was obtained during esterification step.

Heterogeneous acid catalysts have been also used. This is important to avoid problems associated with homogeneous catalysts. Sulphated tin oxide has been used as superacid catalysts to transesterified waste cooking oil [21]. Sulfated zirconias was also used as catalysts in the alcoholysis of soybean oil and in the esterification of oleic acid [20]. Heteropolyacid was used to transesterify yellow horn oil [84]. Anion and cataion

exchange resin was used for triolein transesterification reactions with ethanol to produce ethyl oleate [85].

2.1.3.3 Supercritical alcohol transesterification

Although catalysts play a great role in reducing transesterification time, their presence promotes complications of final product purification. This lead to increase the production process cost.

To avoid catalyst drawbacks, supercritical alcohol (SCA) transesterification process was suggested [39, 47, 86]. SCA transesterification process is a catalyst free process, which is completed in a short time. As a result of catalyst absence, purification of the products of the transesterification reaction is much simpler and environmentally friendly compared to the previous mentioned processes.

In 2001, sake and Kudiana [28] conducted a research on biodiesel production from vegetable oils without any aid of catalysts. That was performed by heating the oil-methanol mixture at the supercritical temperature. Biodiesel was removed from reaction system and excess methanol was removed by evaporating for 20 min at a temperature of 90°C. It was reported that 95% conversion was achieved in the first 4 min of reaction with optimum process parameters of molar ratio of 42:1, pressure of 430 bar and reaction temperature of 350°C. After one year (2002), Demirbas [86] studied transesterification of six different vegetable oils in supercritical methanol.

Comparing with catalytic reactions, SCM reactions were fast and high conversions were reached in very short time. However, the reaction requires higher temperatures, pressures and alcohol to oil molar ratio in comparison to catalytic transesterification, which result in high production cost [59, 60].

Despite the fact that conventional chemical transesterification processes give high conversion, they are usually several drawbacks. It is energy intensive, recovery of by-product is difficult, catalysts have to be removed and waste treatment will be required [7]. To overcome conventional chemically catalyzed biodiesel production methods drawbacks, enzymes have been proposed [87]. Most important advantage of catalyst

enzymes are their ability to convert FFA contained in the fat or oil to methyl esters completely. Additionally, glycerol, by-product, can be easily recovered [39].

2.1.3.4 Enzymatic transesterification using lipase

There is great interest in using biocatalysts to catalyze TG transformation to biodiesel. This has an advantage in having low operating conditions and high purity of the products. Enzymatic transesterification can be carried out at 35 to 45°C [88-90]. Contrary to chemical catalysts, enzymes do not form soaps and catalyze esterification of FFA and TG in one step without any need of washing step. On the other hand, the major disadvantages of the enzymatic transesterification are its slower reaction rate than the chemical catalyst and enzyme inactivation by methanol [57].

An enzyme is a protein molecule whose purpose is to speed up bioreactions. Enzymes have the ability to increase reaction rate by favouring different reaction paths with lower activation energy. In fact, catalyzed reaction takes place in only a small part of the enzyme called the active site [91]. Enzymes are cable of producing products in few process steps, improve product separation and use of low energy [7, 58, and 92]. In general, best enzymes are able to convert more than 90% of the raw material to the desired product at temperature in range of 30 to 50°C.

Lipase is an enzyme able to catalyze methanolysis reactions. It can be obtained from microorganisms such as bacteria and fungi. Lipase from *Mucor miehei*, *Rhizopus oryzae*, *Candida antarctica*, and *Pseudomonas cepacia* are the most commonly used [57, 62]. Lipases belong to a group of hydrolytic enzymes called hydrolases. In biological systems, lipases hydrolyze TGs to fatty acids and glycerol [58]. They work in mild conditions and have an ability to work with TGs from different origins. They have the ability to catalyze transesterification of both TGs and free fatty acids to give esters.

Extracellular and intracellular lipases are the major biocatalyst [56]. Extracellular lipases refer to the recovered enzymes from the microorganism which is then purified, whereas intracellular lipases, the enzyme remains inside the producing cell walls [57]. In term of region-selectivity, lipases have been divided into three types [89].

1. sn-1,3-specific: hydrolyze ester bonds in positions R_1 or R_3 of TG

2. sn-2-specific: hydrolyze ester bond in position R_2 of TG
3. non-specific: do not distinguish between positions of ester

Fjerbaek et al. [62] stated that for biodiesel production from TG , lipases should be non-stereospecific where all TG, DG and MG can be converted to FAME. In addition, they should also be able to catalyze FFA esterification.

Despite the lipases advantages over acid and base catalysts, lipases are costly which limit their industrial use [50, 93]. For that reason, reusability of the enzyme by using it in an immobilized form is essential from the economic point of view.

2.1.3.4.1 Lipase immobilization

In a solution, soluble enzyme acts as a solute in that they are dispersed in the solution and can move freely, but at the same time difficult to separate and to handle. One promising approach to overcome this difficulty is to immobilize the enzyme in a way that can be separated later by any simple separation method. Enzyme immobilization is a technique where freedom movement of the enzyme is restricted and localized to an inert support or carrier. This technique has many advantages; the most important of which is that the immobilized enzyme can be reused [35, 94]. In addition, by immobilization, the operating temperature of the process can be increased [62]. Cao [94] mentioned that an immobilized enzyme has to perform two essential functions: namely, the non-catalytic functions that are designed to aid separation and the catalytic functions that are designed to convert the targeting substrates within a desired time and space. Jegannathan et al. [95] added environment friendly factor as an essential function to achieve sustainability.

Enzyme immobilization can be carried out in different methods. This can be classified into chemical and physical methods as shown in Figure 2.3. In biodiesel enzymatic production, various immobilization techniques have been used. Du et al. [22] used adsorption using macroporous resin, Noureddini et al. [25] worked on hydrophobic sol–gel support by entrapment and Orçaire et al. [26] worked on silica aerogel by encapsulation. Table 2.2 summarizes some of biodiesel previous works using different immobilization methods.

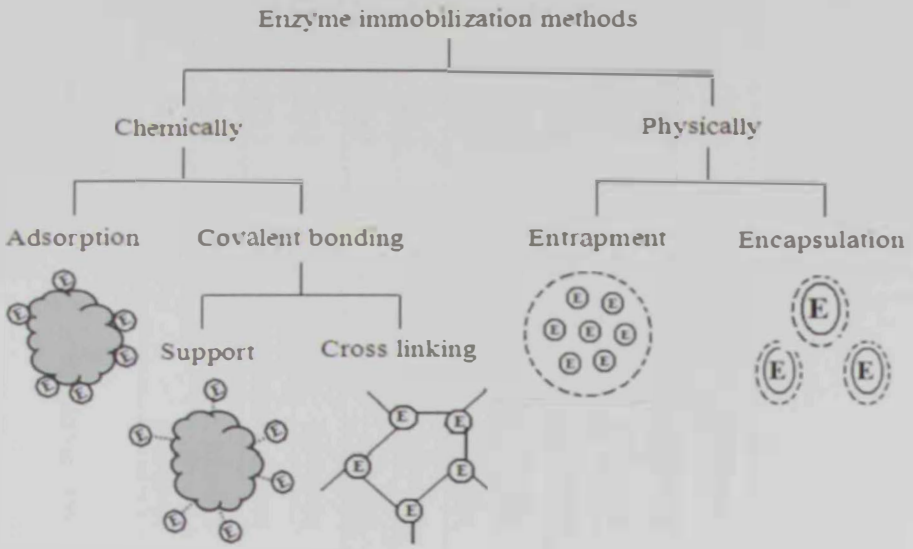

Figure 2.3: Enzyme immobilization methods

Table 2.2: Biodiesel production studies using different lipases immobilized by different methods.

Lipase	Immobilization method	Carrier	Fat / Oil	Acyl acceptors	Yield (%)	Reference
C. antarctica	Adsorption	Silica gel	Soybean	Methanol	94	[96]
	Adsorption	Anion resin	Palm kern	Ethanol	63	[97]
	Adsorption	Acrylic resin	Soybean	Methyl acetate	92	[22]
	Adsorption	Acrylic resin	Soybean	Methanol	92.8	[24]
	Cross linking	Glutaraldehyde	Madhuca	Ethanol	92	[98]
P. cepacia	Adsorption	Celite	Jatropha	Ethanol	98	[27]
	Entrapment	Hydrophobic sol-gel	Soybean	Methanol	56	[25]
	Entrapment	Phyllosilicate sol-gel	Tallow greases	Ethanol	94	[23]
	Encapsulation	Burkholderia cepacia	Sunflower	Methyl acetate	64	[26]
P. fluorescens	Adsorption	Polypropylene EP 100	Sunflower	Methanol	91	[99]
	Adsorption	Polypropylene MP1004	Soybean	Methanol	96	[100]
	Entrapment	Sodium alginate	Jatropha	Methanol	72	[101]

Amongst all possible immobilization methods, physical adsorption has been clearly selected by most researchers due to its ease, the absence of expensive and toxic chemicals, ability to retain the activity and feasibility of regeneration [5]. But, immobilized enzymes are also subjected to diffusion (internal and external) and inactivation (mostly by methanol) limitations [102]. These problems have been studied and pre-solved by different researchers.

2.1.3.4.2 Enzymatic transesterification: Process developments

A number of studies have shown that lipases can be used instead of Conventional chemical catalysts and give great results. Most of these studies focused on reaction condition optimization (temperature, alcohol to oil ratio, used lipase source, lipase to the substrate ratio, reaction time). Additionally, other studies focused on; solvent systems, using different acyl acceptors, acceptor addition steps, lipase pre-treatment and using a combination of different lipases rather than using one lipase.

2.1.3.4.2.1 Methanol step-wise addition

In response to enzyme deactivation by methanol drawback, Shimada et al. [103] suggested that stepwise addition of methanol can reduce methanol inhibition. They mentioned that inactivation problem was due to contact of lipase with methanol which was insoluble in reaction mixture. To avoid this, they suggested conducting first step of the reaction with one third molar of required methanol. After a certain time, second third portion of methanol should be added to the reaction mixture whereas last portion should be added later. In their study, for 48 hr reaction, first portion was added at the beginning of the reaction and after 7 hr of reaction, 33.1% conversion, second portion was added. Conversion of 66.4% was obtained after 10 hr. lastly, after 24 hr of reaction, last portion were added and the reaction was continued for more 24hr. By the end of the reaction, conversion of 97.4% was accomplished. Comparable study was performed by Watanabe et al. [104] for a mixture of oils (soybean and rapeseed) by step-wise addition of methanol where yield of 98.5% was achieved.

2.1.3.4.2.2 Lipase transesterification with and without solvent media

Another suggested solution is the addition of inert solvent that can avoid enzyme inactivation. Addition of solvent is not highly recommended since this will require using of solvent recovery units at the end. Solid fats have a high melting point which is mostly near to lipase denaturation temperature. Addition of solvent as reaction media has the ability to dissolve solid fats, therefore, avoid reaching denaturing temperature [5, 52]. The idea of using solvents was developed by Boocock et al [105]. They produced biodiesel from soybean oil using different acyl acceptors, methanol and butanol with methoxide and sodium butoxide catalysts, respectively. Results showed that when using methanol, produced biodiesel rate was 15 times slower than when using butanol. This was realized to occur as a result of two phase reaction due to low solubility of methanol in oil. To solve this, they suggested using co-solvent like tetrahydrofuran, THF, to produce one phase system.

Köse et al. [106] investigated lipase catalyzed transesterification of cotton seed oil with methanol in solvent-free medium. Yield of 92.1% was achieved in the presence of the Novozym 435 and for 24 hr reaction. This was performed at 50°C, 4:1 molar ratio and 30% enzyme loading. In 2007, Royon et al. [107] used Novozym 435 too for cotton seed transesterification. This was performed at the same condition as Köse et al. but with using *tert*-butanol as solvent. They noted that *tert*-butanol dissolved both of methanol and glycerol that might inhibit enzyme activity. They reported that 97% conversion was observed after 24 hr of reaction in the presence of *tert*-butanol. Nelson et al. [108] tested the effect of using different lipases with different acyl acceptors and systems on biodiesel yield. By using *M. miehei* lipase, yield of 94.8% was obtained in *n*-hexane system, whereas only 19.4 % yield obtained in a solvent free system after 8 hr reaction when using methanol. Ethanol produced 65.5% in solvent free system and 98.0% in *n*-hexane system for only 5 hr reaction.

tert-butanol has been used as solvent in many researches. Li et al. [109] used *tert*-butanol for rapeseed oil transesterification using Novozym 435 and Lipozym TI LM. They reported that a conversion of 95% was obtained. Wang et al. [96] mentioned that when using *tert*-butanol as the reaction medium, negative methanol and glycerol inhibition effects on lipase activity could be eliminated. That is because both of methanol and glycerol are soluble in *tert*-butanol. In their work, they noticed that *tert*-butanol

presence could improve the solubility of methanol in the reaction mixture. Highest yield of 84% was obtained when 80% of *tert*-butanol was used, whereas a further increase in *tert*-butanol decreased the yield. This was justified by dilution effect.

The important of using solvents has been confirmed. However, in order to overcome previous mentioned solvents drawbacks, efforts have been made to offer alternative solvent that is non-toxic and environmentally friendly. Candidate solvents that can replace previous mentioned solvents should have same advantages of dissolving both substrates and reduce excess alcohol inhibition and at the same time avoid the drawbacks of difficult separation of the solvent and toxicity. SCF is one of offered solvents [110] . Further discussion of the use of SC-CO_2 as a reaction medium is found in section 2.2.3.2.

2.1.3.4.2.3 Different acyl acceptors

As mentioned earlier the use of excess alcohol leads to reduce enzyme stability [22, 109]. Abigor et al. [111] found that in the palm kernel oil conversion using *P.cepacia* lipase, ethanol gave the highest conversion of 72% while only 15% methyl ester was obtained with methanol. To overcome methanol inhibitory, different acyl acceptors rather than methanol have been studied. Methyl and ethyl acetates were the most used acceptors for interesterification of different oils and fats into biodiesel. Du et al. [22] performed a comparative study on Novozym 435 transesterification of soybean oil with methanol and interesterification of same oil with methyl acetate for biodiesel production. Methanol showed a negative effect on tested enzymatic activity, whereas methyl acetate did not have any visible negative effects. Biodiesel yield of 92% was obtained at 40°C and 4% Novozym 435 based on oil weight. Methyl acetate with Novozym 435 was also used by Xu et al. [112] to produce biodiesel from soybean oil at 40°C and 92% yield was obtained too. Ognjanovic et al. [113] studied the possibility of producing biodiesel from sunflower oil with different acyl acceptors in a solvent-free system using Novozym 435 lipase. High conversion of greater than 99% was obtained using methyl acetate. Their results showed that high yield of 95.7% has been reached by methanol in the first reaction cycle, but the enzyme activity decreased with run repetition to give 42.3% and 5.1% yield with second and third runs, respectively. On the other hand, methyl acetate yields 99.8%, 98.99% and 98.99%, respectively. Modi et al. [114] produced biodiesel from jatropha, karanja and

sunflower oils using Novozym 435 and ethyl acetate. With their study, obtained yields of ethyl ester were 91.3%, 90.0%, and 92.7%, respectively. Modi et al. [115] reported biodiesel production with Novozym 435 using propan-2-ol as an acyl acceptor with the same oils. Biodiesel yields of 92.8, 91.7 and 93.4% were obtained from jatropha, karanja and sunflower oils, respectively. Despite its clear advantages on enzyme activity, using methyl acetate as an acyl acceptor results in a much slower reaction rate [22].

2.1.3.4.2.4 Lipase pre-treatment

Numerous studies were reported on immobilized lipase pre-treatment effects on its activity and stability. Namakwa et al. [116] studied the effect of pre-incubation of Novozym 435 on soybean oil transesterification with methanol. In their study, immobilized lipase was incubated in methyl oleate for 0.5 hr and then in the oil for 12 hr. When the enzyme was incubated in methyl oleate, 10% product content was achieved after 0.5 hr of incubation and 20% after 1hr, whereas product contents 7.4% and 13.6% were obtained, respectively, when transesterification was carried out without incubation pre-treatment step. Within 3.5 hr of reaction, 97% of product was obtained by step-wise addition of 0.33 equivalent of methanol at 0.25-0.4 hr intervals. Modi et al. [114] also studied similar pre-treatment effect but using ethyl acetate. Results showed that the relative activity of lipase was not affected. Pre-treated lipase gave the yields of 91.1%, 89.6% and 92.4% with jatropha, karanja and sunflower oils, respectively, whereas un-treated lipase yields were 91.3%, 90% and 92.7%, respectively.

2.1.3.4.2.5 Lipase combination

Using two immobilized lipases with opposite specificity position was proposed by Li et al. [117]. Catalyzed methanolysis of rapeseed with a combination of two immobilized enzymes (Novozym 435, non-specific, and Lipozyme TL, 1,3 specific,) in a ratio of 1:3 were tested. This was performed at 35°C with *tert*-butanol solvent system. Conversion of 95% was achieved after 12 hr.

2.1.3.4.3 Enzyme kinetic

The study of enzyme kinetics is important. Enzyme kinetics mean analysis (quantitatively) of the factors that determine enzyme catalytic activity. First proposed study of enzyme catalytic potential was performed in 1903 by Henri, who studied the effect of substrate concentration. Henri investigated that substrate conversion to product proceed in a reversible reaction between the substrate and enzyme to form an intermediate that breaks down to deliver the product.

In 1913, Henri's ideas were used by Michaelis and Menten, who started the first formal theory for enzyme catalysis on a single substrate. After a while, Michaelis and Menten equation were established as shown in Eq. (2.1) . Eq. (2.1) represents the parametric expression for the enzymatic reaction rate as a function of single substrate concentration. K_m represents Michaelis constant, [S] represents substrate concentration, *and* v represents initial reaction velocity while v_{max} represent maximum reaction velocity. This is a case a single substrate involved in the reaction without any inhibition either from an inhibitor, substrate or product to the enzyme.

$$v = \frac{v_{max}[S]}{K_m + [S]} \qquad \text{Eq. (2.1)}$$

In some of enzymatic reactions, presence of excess substrate inactivates the enzyme by binding with formed intermediate. For this case, the following model Eq. (2.2) was proposed.

$$v = \frac{v_{max}[S]}{K_m + [S]\left(1 + \frac{[S]}{K_{IS}}\right)} \qquad \text{Eq. (2.2)}$$

For the enzymatic reactions discussed so far, it was assumed that only one substrate present. However, in most enzymatic reactions, two or more substrates may take palace, Still, Michaelis–Menten valid for this as long as the dependence of only one substrate is studied and the other substrate is kept in a large amount. If two substrates are

being converted into one single product, mechanism is called a bi-uni mechanism while if two substrates are being converted into two different products, mechanism is called bi-bi mechanism. Mechanisms where all substrates must bind before the product can being released are called sequential mechanisms. On the other hand, mechanism where the product is being released or formed before all substrates being bound to the enzyme is called ping-pong mechanism.

Many authors verified that kinetic mechanism for transesterification reaction follows ping-pong mechanism with competitive inhabitation of an alcohol [118-121]. General ping-pong reaction rate with alcohol competitive inhabitation is shown in Eq. (2.3). $[S_1]$ and $[S_2]$ are TG and alcohol substrates concentration respectively, K_1 ,K_2 are Michaels constants while and K_{IS2} is substrate inhibition constant.

$$v = \frac{v_{max}[S_1][S_2]}{[S_1][S_2] + K_2[S_1] + K_1[S_2]\left(1 + \frac{[S_2]}{K_{IS}}\right)} \qquad \text{Eq. (2.3)}$$

2.1.4 Possible Feedstocks

Biodiesel can be synthesized from a great variety of feedstocks. These feedstocks include most vegetable oils (soybean oil, jatropha oil, rapeseed oil, palm oil, sunflower oil, corn oil, peanut oil, canola oil and cottonseed oil) and animal fats (tallow and lard). Besides, can also be produced from other sources like waste cooking oil, greases and algae [44].

Oils and fats belong to a family of chemicals called lipids, which are found in animals and plants. In general, fats come from an animal source whereas oils from a plant source. These two are primarily formed of TG molecules. A TG molecule is mainly made of one mole of glycerol and three moles of fatty acids [6, 39]. The TG of vegetable oils and animal fats typically contain several different FA. Thus, different FA can be attached to one glycerol backbone. Because different FA have different physical and chemical properties, the FA composition is probably the most important parameter influencing the corresponding properties of a vegetable oil or animal fat [87].

2.1.4.1 Vegetable oils

Possibility of using vegetable oils as a fuel has been known from the time where diesel engines known. This was in 1900 when Rudolf Diesel used groundnut oil [2, 66, 87, and 122]. Vegetable oils had been used for an emergency conditions such as World War 2 as fuel to run the engine [54, 123]. Mainly, vegetable oils consist of the TG of the straight chain FA. The FAs which are commonly found in vegetable oils are stearic, palmitic, oleic, linoleic and linolenic.

Since vegetable oil is a feedstock that is available in large quantities, it has been widely used for the conversion to biodiesel. Majority of vegetable oils have been employed for biodiesel production via transesterification process such as soybean oil [124, 125], rapeseed oil [28, 109], canola oil [126], palm oil [127-129] and sunflower oil [130, 131].

2.1.4.2 Waste cooking oils

Waste vegetable oils and fats are a product of repeatedly uses vegetable oils and fats from cooking and frying processes. Fried oils and fats are usually broken down after a period of use and become un-suitable for further cooking as a result of increasing of free fatty acid content. Once this reached, they are discarded or recycled. This type of feedstocks is of low cost and can solve the high feedstock cost problem [132]. Waste cooking oil conversion into biodiesel through the transesterification process approximately reduces the molecular weight to one-third, viscosity by about one-seventh, flash point and volatility [39, 133].

2.1.4.3 Algae

Using microalgae oil as a biodiesel feedstock is not new. In 1980, Nagle and Lemke [134] examined several solvent systems for lipid extraction from microalgae and identified the impact of major variables in microalgae transesterification. Using microalgae oil for biodiesel production has many interesting features [135]. These include

that the CO_2 exhaust from coal-burning power plants can be used as a carbon source for the microalgae, the residual biomass after oil extraction can be used for ethanol production [102]. Among known algae, diatoms (Bacillariophyceae), green algae (Chlorophyceae) and blue-green algae (Cyanophyceae) are the most common groups of algae targeted for biodiesel production. Therefore, there is a great interest in producing biodiesel from algae where it does not compete with food production as vegetable oils [136-138]. Hossain and Salleh [135] produced biodiesel from algae transesterification and promised results were obtained. Table 2.3 summarizes some of enzymatic biodiesel production using different feedstocks, lipase, acyl acceptors, reaction solvents, operating conditions and lipase form.

2.1.4.4 Animal Fat

Animal fats are received from cattle, hog, chicken, lamb and fish. Tallow's and animal meats which are not allowed to be used as food can be used as biodiesel feedstock. Moreover, these two sources have discontinuity supply problem. It is possible that suddenly a high bulk of material is available followed by a period with no supply like what is happening in case of animal disease [139]. Animal fats are characterized by the high amount of saturated fatty acids (SFA). They are solid at room temperature and cannot be used as fuel in a diesel engine in their original form [140]. In this study, fat extracted from animal meat was used as a triglyceride source. This is difficult to be obtained using conventional chemical extraction, leading to the requirement of SC-CO_2 extraction. Section 2.3 gives more details animal fat extraction. The novelty of this work is the use of waste animal fats for biodiesel production, while leaving healthy low fat meat. This has not been so far investigated.

Table 2.3: Summary of Enzymatic production of biodiesel from different feedstocks, acyl aceptors, solvents, lipase form and reaction conditions.

Lipase	Fat/Oil	Alcohol	Solvent	Temperature (°C)	Molar ratio (Alcohol: oil)	Reaction time	Lipase form	Yield (%)	Reference
C. antarctica	Cotton seed	Methanol	*tert*-butanol	50	4:1	24 hr	Imm.	95	[141]
	Rapeseed	Methanol	*tert*-butanol	35	4:1	12 hr	Imm.	95	[109]
	Jatropha	Ethyl acetate	Free	50	11:1	12 hr	Imm.	91.3	[142]
	Soybean	Methanol	Ionic liquid	50	4:1	12 hr	Imm.	80	[143]
	Soybean	Methyl acetate	Free	40	12:1	14 hr	Imm.	92	[144]
	Soybean	Methyl acetate	Free	40	12:1	14 hr	Imm.	92	[22]
	Palm kern	Ethanol	SC-CO_2	40	10:1	4 hr	Imm.	63	[97]
	Sunflower	Methanol	SC-CO_2	45	5:1	6 hr	Imm.	23	[29]
	Sunflower	Ethanol	SC-CO_2	45	5:1	6 hr	Imm.	27	[29]
P. fluorescens	Sunflower	1-propanol	Free	60	3:1	20	Imm.	91	[35]
P.cepacia	Tallow + Grease	Ethanol	Free	50	4:1	20	Imm.	94	[23]
	Jatropha	Ethanol	Free	50	4:1	12 hr	Imm.	98	[145]
	Sunflower	2-butanol	Free	40	3:1	6	Imm.	100	[146]
	Madhuca	Ethanol	Free	40	4:1	2.5	Imm.	92	[98]
	Soybean	Methanol	Free	35	7.5:1	30	Imm.	56	[25]
	Mahua	Ethanol	Free	40	4:1	6	Imm.	96	[98]

2.2 SUPERCRITICAL FLUIDS

Any substance at a temperature and pressure above their critical values is referred to as a SCF. In 1822, Baron Cagniard discovered supercritical fluids and observed that the boundary between a gas and a liquid disappears with increase in temperature. However, it was not until 1869 when Andrewa discovered the existence of this new state of matter. In 1879, Hannay and Hogarth investigated the solvating power of SCF, namely they studied solubilities of cobalt (II) chloride, iron (III) chloride, potassium bromide and potassium iodide in supercritical ethanol [147]. Since then supercritical fluid technology has been widely used in extraction and purification processes in food and pharmaceuticals industries as well as in organic synthesis [148].

In this section, a review of some of the basic fundamentals of SCFs and related physical and chemical properties with special focus on supercritical CO_2 (SC-CO_2) are introduced, followed by several of applications areas of SCF. Major part is centred on using supercritical fluids, especially SC-CO_2, as an extraction solvent and reaction media.

2.2.1 Fundamentals of SCF

Supercritical fluids, SCFs, are substances at pressures and temperatures above their critical values, P_c and T_c respectively. Critical values represent the highest temperature and pressure at which the substance exists as a vapour and liquid in equilibrium. This can be simply clarified from supercritical fluids phase diagram (Figure 2.4).

As shown in Figure 2.4, there are three single phases where a substance may occur as a result of either temperature or pressure. The three phases are solid, liquid and gas. If a mixture of two, or more, phases exists in these regions, a separation between the phases is distinct, as a result of the differences in properties of the different phases. In Figure 2.4, the solid curves between phases indicate the co-existence of two phases. So, solid-gas, solid-liquid and liquid-gas curves correspond to sublimation, melting and vaporization, respectively. These three curves intersect at a point where the three phases co-exist together. This point is referred to as the triple point. On the other hand, at a point beyond the critical neither liquefaction will be taken place as a result of pressure increase, nor gas

will be formed as a result of temperature increase. This is what was defined earlier as a supercritical region. Table 2.4 lists values of the critical temperatures and pressures of different substances [147].

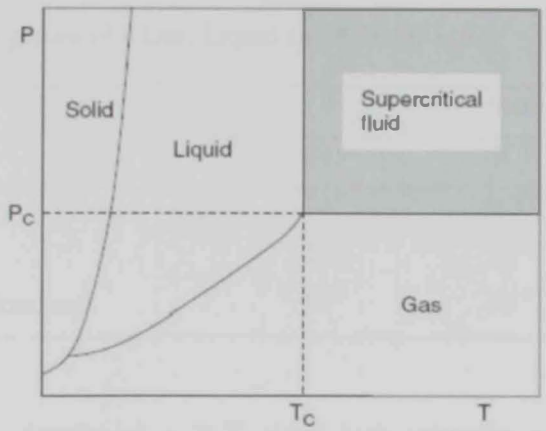

Figure 2.4: Pure component phase diagram [149]

Table 2.4: Critical data of some common solvents used in SCF sate [150].

Substance	Critical Temperature (°C)	Critical Pressure (bar)
Xenon	16.7	59.2
Carbon dioxide	31.1	72.8
Ethane	32.4	49.5
Nitrous oxide	36.6	73.4
Chlorodifluoromethane	96.3	50.3
Ammonia	132.4	115.0
Methanol	240.1	82.0
Water	374.4	224.1

In the SCF state, a solvent displays properties which are intermediate to those of liquid and gaseous states, SCFs have more desirable transport properties than liquids and better solvent properties than gases. A general comparison between the physical properties of gases, liquids and SCFs is presented in Table 2.5. Listed data demonstrates

that the viscosity of supercritical fluid is similar to that of a gas while diffusivity lies between that of a gas and a liquid.

Table 2.5: Physical Properties of a Gas, Liquid and SCF [38, 151]

Property	State		
	Gas	SCF	Liquid
Density (g/cm^3)	10^{-3}	0.3	1
Diffusivity (cm^2/sec)	0.2	10^{-3}	10^{-5}
Dynamic Viscosity (g/cm.sec)	10^{-4}	10^{-4}	10^{-2}

The liquid-like density of a SCF gives high solvation power and facilitate solubility while the gas-like diffusivity gives excellent transport properties, which increases the rates of transfer from the substrate matrix to the SCF solvent as compared to that of liquid organic solvents [148]. Moreover, the low viscosity of SCFs which is close to that of the gases is an additional advantage. This last property gives rapid solvent penetration into a solid matrix [152].

2.2.2 Applications

In the last 20 years or more, SCF technology applications have been mostly in extraction and chromatography purposes. However, nowadays SCFs have been used for a wider range of applications [147]. Interesting SCFs properties make them good solvents for several applications. In the last few decades, numerous researches have been made in order to utilize the full potential of SCF's in separation and reaction applications. Examples of SCF extraction applications include coffee and tea decaffeination, flavours from hops, cholesterol and fat from eggs, nicotine from tobacco, acetone from antibiotics, and organics from water [151].

2.2.3 Supercritical Carbon Dioxide as a candidate solvent

Even though a number of substances could serve as solvents, CO_2 is the most favourable to many applications. SC-CO_2 ability to extract a solute depends on the compounds functional groups, molecular weight and polarity. Near to critical point, CO_2 is a good solvent for non-polar to slightly polar solutes with low molecular weight. It is an inert at most conditions, inexpensive, non-toxic and environment-friendly solvent [38]. Moreover, when using SC-CO_2 as a solvent no solvent residue remains in the extract as it is in a gas phase at the ambient conditions [153]. The critical temperature and critical pressure of CO_2 are 31.1°C and 72.8 bar, respectively, which are highly not extremely high. SC-CO_2 has been well-known as a good alternative solvent for a number of lipid processing operations including the separation of FA from animal lipids [154].

2.2.3.1 SC- CO_2: Extraction solvent

Extraction is the process of removal of a solute from a matrix using a solvent which is able to dissolve the desired solute. This involves contacting the matrix with the solvent either in a single stage or in multiple stages for certain period of time and then separating the solvent. During extraction period, solute will be transferring from the matrix to the solvent. Required time to achieve successful extraction depends on the solubility of the solute in the solvent. That depends on extraction temperature, contact area between the solute and solvent, solvent viscosity as well as solvent flow rate [43].

Solvent extraction is one of the conventional extraction techniques. Using this conventional technique presents some drawbacks such as long extraction time, high solvent consumption, labour intensive, difficult to automate, use of toxic solvents and often require a post-extraction clean-up [155]. With these drawbacks, supercritical fluid extraction, SFE, has been proposed using the extraction solvent in its supercritical state.

SCFs were first observed more than a century ago, 1822. However, SFE has been developed as a novel separation technique only in the past two or three decades. SFE is an extraction process which is carried out using SCF as a solvent. SCFs are mostly used as an extract solvent in the approximate range of temperature up to 1.2 times the critical temperature, T_c, and pressure up to 3.5 times the critical pressure, P_c [156].

Typical SFE system consists of high pressure pump that delivers the fluid and an extraction cell that contains the sample and maintained at the desired temperature and pressure. Additionally, SFE may consist of a modifier pump too. Modifiers are usually used to enhance fluid solvation properties. SFE can be operated in two modes: static or dynamic. In static mode, SCF is held in an extraction cell for a certain time and then released to a collection device. In dynamic mode, the SCF flows continuously through the extraction cell and out into a collection vial.

Chlorodifluoromethane (Freon-22 or R22) has been used to extract steroids [157] and soil organic matter [158]. Additionally, nitrous oxide (N_2O) has been used to extract taxol from Taxus brevifolia [159]. However, these fluids application is limited as a result of undesired environmental properties of the solvent. Raynie [160] reported that N_2O caused an explosion and destruction of the extraction vessel when it was used as a SFE solvent for ground coffee samples and Freon-22 has negative effect on ozone.

Even though a number of substances could serve as solvents, CO_2 is the most common. SC-CO_2 has many applications, especially in food processing, which include decaffeination coffee and tea, production of hops extracts, flavours extract from herbs and extraction of edible oils. Friedrich et al. [92] extracted oil from soybeans using SC- CO_2. Oil yield of 18.3% was obtained from soybean oil compared to a yield of 19.0% was obtained using *n*-hexane. In order to extract polar compounds from a matrix, polar supercritical fluid should be used. Thus, CO_2 sometimes face difficulties to extract certain compounds from a sample matrix. To solve this, modifier fluids can be used to increase extraction efficiency. Selected modifier should be able to increase desired compound solubility. Various modifiers have been tried. Among all the modifiers, methanol is the most commonly used by various investigators. Tonthubthimthong et al. [161] extracted nimbin from neem seeds. Brewer et al. [162] extracted cocaine from human hair and Aghel et al. [163] extracted pennyroyal essential oil using SC-CO_2 with methanol as modifier.

Co-solvents use has been studied widely where its impact on the solubility has been focused. Several reports have successfully employed ethanol as a modifier in SFE from plants. Hardardottir and Kinsella [164] investigated % cholesterol reduction in fish muscle using SC-CO_2 with 10% ethanol. Using pure SC-CO_2, at 275 bar and 40°C, 97% reduction of cholesterol was obtained after 9 hr of extraction, whereas using SC-CO_2 with

10% ethanol allowed 99.7% cholesterol reduction in only 6 hr. Ooi et al. [165] investigated refining crude palm oil where oil content was reduced from 2.35 to 0.19% at 240 bar and 50°C. On the other hand, 3.7 mol% ethanol as a co-solvent addition to SC CO_2 reduced the content to less than 0.1% at 206 bar and 50°C. With a further increase of ethanol to 6.3 mol% to SC CO_2 solvent at 171 bar and 50°C content was reduced to 0.04%.

2.2.3.2 SC- CO_2 : Reaction media

Majority of chemical processes are carried out in organic solvents. From the environmental point of view, most of these solvents are toxic and flammable. Furthermore, they have to be separated from the desired product and recycled back. To avoid these, SCFs can be used as an alternative .

As mentioned earlier, SCFs have gas-like diffusivities and low viscosities which reduce mass resistance between reaction mixture and the catalyst. Therefore, result in an increase of reaction rate. Among possible solvents that can be used in supercritical conditions to conduct transesterification reactions, carbon dioxide was chosen due to its low critical temperature.

Kumar et al. [88] esterified palmitic acid with ethanol in temperature range of 35 to 70°C in the presence of three different lipases in SC-CO_2. Their results showed that Novozym 435 was the best catalyst. In SC- CO_2, Lipolase 100T and hog pancreas lipase showed similar results. Yields of 74, 44 and 40% were reached using Novozym 435, Lipolase 100T, and hog pancreas lipase, respectively, whereas in solvent free system yields of 97, 66 and 40% were obtained. Romero et al. [110] esterified isoamyl alcohol in SC- CO_2 and *n*-hexane. They noted that similar esterification degree was obtained in both SC- CO_2 and *n*-hexane systems; however, initial reaction rate was higher in SC- CO_2. In 2007, laudani et al. [166] compared FFA esterification with 1-octanol over immobilized lipase from *R. miehei* (Lipozyme RM IM) using three different reaction media: SC-CO_2, *n*-hexane and solvent free systems. SC-CO_2 showed the highest conversion followed by *n*-hexane and solvent free system.

Limited works were done on transesterification , among them, Oliveira and Oliveira [97] compared enzymatic alcoholysis of palm kernel oil using *n*-hexane and SC-CO_2 systems. In SC-CO_2, highest conversion of 63.2% was obtained using Novozym 435 as catalyst whereas in *n*-hexane Lipozyme IM provided the highest conversion of 77.5%. In 2004, Rathore et al. [51] produced biodiesel from Jatropha oil with Novozym 435 in SC- CO_2. Optimum conditions was found to be 45°C, 5:1 molar ratio,30% enzyme loading and 8 hr with conversions of 60-70%. In 2007, Varma et al. [120] produced biodiesel from caster and linseed oils with Novozym 435 in SC- CO_2 where 45% yield in methanol and 35% in ethanol were obtained from linseed oil while a very low yield, less than 10%, was obtained from caster oil. Varma et al. [167] synthesized biodiesel from mustard and sesame oils using different acyl acceptors at 50°C for 24 hr reaction. Their results showed that using mustard oil, conversion of roughly 70% and 65% obtained using methanol and ethanol, respectively. On the other hand, using sesame oil a conversion of round 55% was obtained from ethanol whereas only 45% obtained with methanol.

2.3 MEAT

With the industrial development and the modern life, many people suffered from obesity caused by eating large amounts of fat foods (either from animal or plant sources). Meat is a major source of fat in the diet, so many people decided to stop meat eating in order to avoid any obesity. On the other hand, meat is an important source of high value of protein.

2.3.1 Meat sources

Meat needs can be obtained from different sources such as; beef (Cattle), sheep (lamb and mutton), goat, camel, poultry (chicken and duck), fish [168]. These animals' meats can be divided into red meat, white meat and processed meat. Red meat includes beef, lamb, veal and pork whereas white meat includes chicken and turkey and processed meat includes ham and hamburgers. Red meat contains high biological value protein and important micronutrients such as Vitamin A, B1 and B2 that are required for good health

life as well as fats [169]. Williams [169] mentioned that there is a wide variation in the amount of total separable fat between the lamb cuts, ranging from 37% in loin lamb chops to only 1% in veal steak as shown in Table 2.6.

Table 2.6: Composition of lean muscle tissue of meat animals [170].

Species	Water	Protein	Lipid	Ash
Beef	70-73	20-22	4.8	1
Chicken	73-7	20-23	4.7	1
Lamb	73	20	5-6	1.4
Pork	68-70	19-20	9-11	1.4

2.3.2 Meat fat and fatty acid content

Meat and meat products vary in their fat content and fatty acid content according to the animal species, age, sex and diet used. Additionally, fat and FA content is also affected by animal feeding [171]. Meat fat comprises mostly saturated fatty acids (SFA) monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFAs). In addition to conjugated linoleic acid (CLA) that has beneficial effects on health, especially in lamb and beef [172, 173]. Connor [174] reviewed importance of n-3 fatty acid in health. Beside fatty acids, cholesterol is a nutritionally important component of meat [171]. The saturated chain would have single bonds between carbons while the unsaturated have one or more double bonds between carbons. There can be one or more double bonds in an unsaturated fatty acid (UFA) [169]. UFAs with only single bonds are called MUFA while a fatty acid with more than one double is called PUFA [175]. Raes, Smet and Demeyer [173] mentioned that PUFA content in TGs may vary between 2 and 30 g/100 g of total fatty acids. Table 2.7 shows common fatty acids presents in fats.

In meat, fats are divided into three major groups; Intramuscular fat (marbling fat) which present in the intramuscular adipose tissue and in the muscle fibres, IMF which present between individual muscles and Subcutaneous fat which present under skin [173]. The difference in TGs can be attributed to the alkyl group of the fatty acids.

William [169] mentioned that the amount of saturated fat is in Australian lamb is actually lower than the total amount of unsaturated fats (Table 2.8).

Table 2.7: Chemical structure of common fatty acids presents in lipids [176].

Fatty Acid	Structure	Formula
Lauric	$C_{12:0}$	$C_{12}H_{24}O_2$
Myristic	$C_{14:0}$	$C_{14}H_{28}O_2$
Palmitic	$C_{16:0}$	$C_{16}H_{32}O_2$
Palmitoleic	$C_{16:1}$	$C_{16}H_{32}O_2$
Stearic	$C_{18:0}$	$C_{18}H_{36}O_2$
Oleic	$C_{18:1}$	$C_{18}H_{34}O_2$
Linoleic	$C_{18:2}$	$C_{18}H_{32}O_2$
Linolenic	$C_{18:3}$	$C_{18}H_{30}O_2$
Arachidic	$C_{20:0}$	$C_{20}H_{40}O_2$
Archidonic	$C_{20:1}$	$C_{20}H_{38}O_2$
Behenic	$C_{22:0}$	$C_{22}H_{44}O_2$
Erucic	$C_{22:1}$	$C_{22}H_{42}O_2$

Table 2.8: Fatty acid profile of raw lean meats (g/100g edible portion) [169].

Fatty acid	Beef	veal	lamb	Mutton
$C_{14:0}$	0.096	0.034	0.101	0.060
$C_{15:0}$	0.012	0.006	0.016	0.011
$C_{16:0}$	0.607	0.215	0.842	0.667
$C_{17:0}$	0.028	0.009	0.051	0.036
$C_{18:0}$	0.356	0.119	0.644	0.609
Total SFA	1.149	0.409	1.730	1.464
$C_{14:1}$	0.025	0.007	0.004	0.003
$C_{16:1}$	0.082	0.033	0.066	0.039
$C_{18:1}$	1.103	0.356	1.995	1.370
$C_{20:1}$	0.015	0.048	0.010	0.011

Total MUFA	1.205	0.399	2.066	1.413
$C_{18:2}$	0.204	0.090	0.321	0.339
$C_{18:3}$	0.048	0.022	0.072	0.107
$C_{20:3}$	0.020	0.012	0.009	0.009
$C_{20:4}$	0.076	0.056	0.094	0.101
$C_{20:5}$	0.031	0.028	0.028	0.044
$C_{22:5}$	0.051	0.033	0.044	0.053
$C_{22:6}$	0.006	0.003	0.013	0.020
Total PUFA	0.448	0.259	0.603	0.673

2.3.3 Meat defatting and Decholesterolification using SC-CO_2

Despite the fact that the red meat is a rich source of protein, it might cause heart diseases as a result of high fat content. Therefore, people started to stop red meats eating. In 1993, Jr [177] mentioned that between 1970 to 1990 period red meat consumption in US was declined to almost 12% where lamb meat consumption fell by almost 40% while chicken, red meat, consumption increased b y 64%. Also, in 1988 Geoffh [178] mentioned that in UK lamb meat consumption reduced from 9.0 kg carcass to 6.6 per capita. Accordingly, lean meats that reduce market meats fatness were introduced. As mentioned previously, meats contain fats as visible fat and IMF. In fact, meat processors trim surface fat but the IMF are outside their control. To overcome this fat extraction where suggested.

SC-CO_2 has used to extract fat from animal sources such as bone meal and a process for deodorising animal fats [179]. Extraction has also been used to remove lipids and cholesterol from fish muscle [164, 180] and fried-shredded pork [181]. In general, SC-CO_2 extraction efficiency largely depends on the quantity of lipids, moisture, lipoprotein, and proteophospholipid complexes present in the meat. By proper selection of the process conditions of SC CO_2 extraction and fractionation, it is possible to fine tune the selectivity of separation of cholesterol and lipids simultaneously in order to get the best possible final product [38].

In meat products, SC-CO_2 has been used as extraction solvent to extract fat and cholesterol. Cholesterol was extracted from dehydrated cooked powdered and chunked

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beef by Wehling et al.. Wehling et al. [182] observed that 85-87% extraction yield of cholesterol at 55°C and 381 bar. Chao [183] explored the feasibility of using extraction technique to alter the cholesterol content of ground meat and edible beef tallow using various extraction methods. King et al. [184] used SC- CO_2 to extract fat from different meat samples at 80°C. Homogenized ham-based luncheon meat, smoked ham, and low-fat import ham were used in their study. Within 1 hr of the extraction period and at 34.5 MPa, yield of over 96% of the theoretical fat content was extracted from the samples. King et al. [185] compared SC-CO_2 effectiveness in reducing cholesterol and fat content of precooked, fresh and dehydrated beef patties. Additionally, Berg et al. [155] investigated the possibility of replacing the Bligh and Dyer extraction with SFE for meat products lipase class determination. In comparison to solvent extraction based method, King et al. [186] developed SFE method to replace current solvent based extractions of fats from meat. MacLachlan [187] mentioned that meat defatting involve three steps: particle size reduction, moisture removal and fat extraction using SC-CO_2 in temperature and pressure ranges that will not affect meat properties.

CHAPTER THREE: MATERIALS AND METHODS

CHAPTER THREE: MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Meat samples

Lamb meat samples from different sources and cuts, namely Australian shoulder, Australian leg and Indian leg, were purchased from the local market and the outer surface fat was skimmed. At all times, samples were stored below -20°C until used.

3.1.2 Chemicals

Liquefied CO_2 with a purity of 99.95% was supplied by Abu-Dhabi Oxygen Company, UAE. Nitrogen (N_2), Hydrogen (H_2) and zero-air with ultra high purity (UHP) were supplied by Sharjah Oxygen Company, UAE. *n*-hexane solvent 99% reagent grade and HPLC grade methanol of 99% assay were obtained from Riedel-de Haën, Germany. 14% BF_3-methanol mixture was obtained from Sigma Aldrich, USA.

Reference standards of high purity percent (as external standard) of methyl ester of 4.0% myristic acid ($C_{14:0}$), 10.1% palmitic acid ($C_{16:0}$), 6.0% stearic acid ($C_{18:0}$), 35% oleic acid ($C_{18:1}$), 36.0% linoleic acid ($C_{18:2}$), 2.0% archidonic acid ($C_{20:0}$) and behenic acid ($C_{22:0}$) were obtained from Sigma Aldrich, USA.

3.1.3 Enzymes

Immobilized *C. antarctica* lipase B supported on macro-porous polyacrylic resin, which is commercially known as Novozym ® 435, having an enzyme activity of 7000 PLU per gram and water content of 2% was obtained from Novozyms A/S, Denmark. The enzyme was stored below 8°C and above 0°C according to the supplier's instructions.

3.2 METHODS

3.2.1 Sample preparation

Before performing any extraction, moisture content in meat samples was reduced to a low level to minimize the negative effect of water in the sample matrix on fat extraction and avoid plugging problems. To study the effect of drying methods on extraction yield, samples were dried using freeze drier (Telstar, Terrassa, Spain) and vacuum oven (Shel-Lab, Sheldon Manufacturing Inc., USA). Freeze drier was operated at -80°C for 6 hrs whereas vacuum oven was operated in 0.02 mbars and 70°C for 24hr. De-moisturized samples were grounded mechanically using a blender (Moulinex, France) for few seconds to decrease particle size and homogenize the sample before extraction.

3.2.2 Fat Extraction

3.2.2.1 Soxhlet extraction

Total fat content of the samples was determined using soxhlet extraction system and a multi-unit extraction heater (Lab-line instruments, Inc., Melrose Park Illinois, USA) using *n*-hexane as solvent. Approximately, 10 g of sample was placed in a soxhlet thimble and then introduced into the soxhlet extractor with about 250 ml of hexane. The system was then heated up causing the fat to be extracted from the samples, until totally exhausted. After 6 hrs of continuous extraction, extracted samples were dried in an oven (Memmert, Germany) and heated to 100°C to remove residual solvent. This procedure was performed for the three different meat sources. Differences in the weight of each sample before and after the extraction process were used to determine the total fat content.

3.2.2.2 Supercritical carbon dioxide (SC-CO_2) extraction

SC-CO_2 extraction experimental apparatus consisted of CO_2 cylinder with a dip tube, CO_2 high pressure syringe pump with a maximum operating of 500 bars (Model 260D, ISCO, USA); pump controller (ISCO, SFX 200, USA) and SFE extraction unit

(ISCO, SFX 220, USA). The dip tube allows only liquefied CO_2 to be transferred to the pump as the liquid resides in the bottom of the cylinder and the gaseous CO_2 at the top.

Extraction unit consisted of an extraction chamber with two 10 ml stainless steel cells and a temperature controlled incubator of maximum 150°C (ISCO, SFX 220). Pump controller that has a control panel displaying time, pressure, CO_2 flow rate as well as volume of CO_2 passed. Pressure within the chamber was measured and controlled by the system whereas the temperature was measured and controlled in the incubator. The precision of the temperature measurements of the extraction system was ±0.1°C. To facilitate having a good control of flow rate, micrometer valve was used. Moreover, water bath with heating coil surrounded the CO_2 cylinder to facilitate CO_2 movement upward. A thermocouple was connected to the temperature controller (Omega CN9000A) to control the temperature on the surface of the micrometer valve (HIP 15-12AF1-V). A schematic diagram of SFE apparatus used is shown in Figure 3.1. Figure 3.2 shows a photograph of the system.

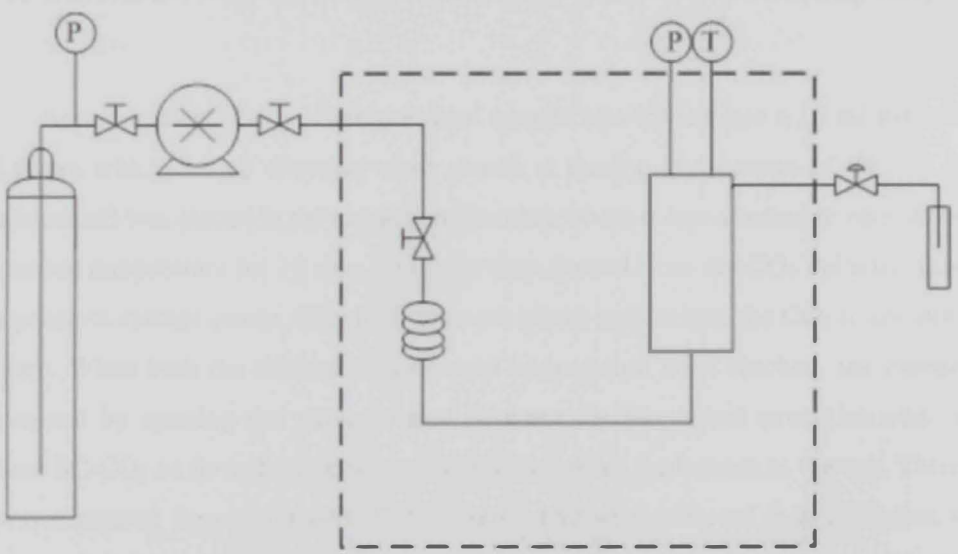

Figure 3.1: Schematic diagram of supercritical carbon dioxide extraction apparatus (1: CO_2 cylinder with dip tube, 2: Pump, 3: heating coil, 4: Extraction cell, 5: Incubator, 6: Metering Valve, 7: sample collection vial)

Figure 3.2: Supercritical carbon dioxide extraction system photograph

(1: CO_2 cylinder, 2: Syringe pump, 3: Incubator, 4: Pump controller, 5: Metering Valve)

Approximately, 2.4 g of pre-prepared sample was loaded into a 10 ml extraction. Cell filters with 5/8 inch diameter were placed at the top and bottom of the cell. The extraction cell was placed in the extraction chamber where it was allowed to equilibrate to the desired temperature for 15 min. CO_2 was then flowed from the CO_2 cylinder into the high pressure syringe pump. This high pressure pump pressurized the CO_2 to the desired pressure. When both the desired pressure and temperature were reached, the extraction was started by opening the valve located between the pump and extraction cell. This allowed SC-CO_2 to flow through the extraction cell from the bottom to the top. Then the fat was separated from extract-SC-CO_2 solution and was collected in a collection vial. The extracted fat was weighted using an analytical balance with precision of 0.0001g (Model XB220A, Precisa, Switzerland) exactly after each 20ml passed CO_2 for all experiments. After each run, system has been flashed first using *n*-hexane followed by SC-CO_2.

The extraction yield was determined based on the cumulative mass of the extracted fat divided by the weight of initial prepared sample as shown in Eq. (3.1).

Extraction efficiency was determined based on mass of the extracted fat divided by total fat content of meat sample, which was determined using soxhlet extraction system as shown in Eq. (3.2).

$$\text{Fat yield}\,(\%) = \frac{\text{Weight of extracted fat}}{\text{Initial sample weight}} \times 100\% \qquad \text{Eq. (3.1)}$$

$$\text{Efficiency}\ (\%) = \frac{\text{Weight of extracted fat}}{\text{Total fats present in the sample}} \times 100\% \qquad \text{Eq. (3.2)}$$

The above procedure was performed for different temperatures in the range of 35 to 55 °C, pressures in range of 300 to 500 bars and average CO_2 flow rates of 3.0±0.5 to 5.0±0.5 ml min^{-1}.

3.2.3 Enzymatic transesterification in SC-CO_2

Enzymatic transesterification system consisted of CO_2 cylinder, CO_2 high pressure syringe pump (Model 260D, Teledyne ISCO, USA), pump controller (D-series, Teledyne ISCO, USA) and reaction cell (SS-316). Pump controller was equipped with a control panel which displayed reaction time and pressure. Reaction temperature was controlled by a temperature controller (Thermolyne Type 45500 input controller, Barnstead) with an electrical heating tape (Thermolyne, USA) wrapped around the reaction cell. A thermocouple connected to a 12-channel thermocouple scanner (Model 692-020, Barnant, USA) was used to read reaction cell surface temperature. Additionally, two valves were used for SC-CO_2 flowing and final product elution purposes. Figure 3.3 shows the experimental setup of enzymatic transesterification of the extracted fat to biodiesel using SC- CO_2 as solvent. Figure 3.4 shows a photograph of the system.

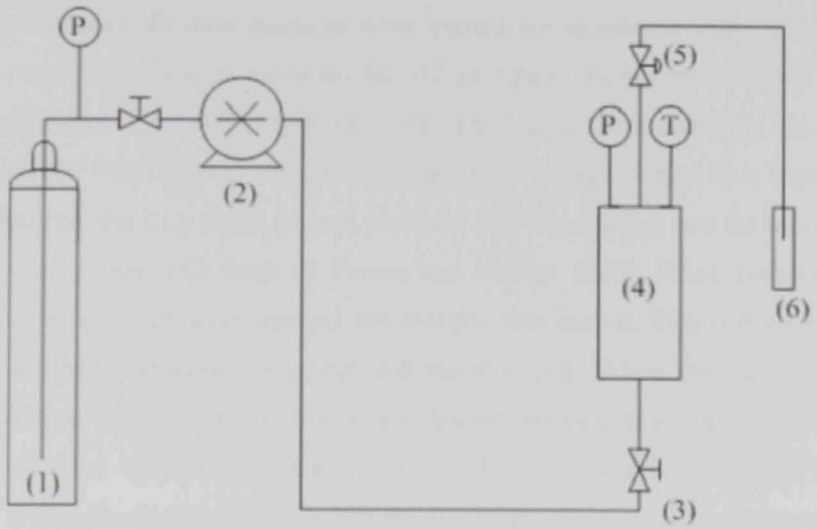

Figure 3.3: Schematic diagram of supercritical carbon dioxide extraction apparatus

(1: CO_2 cylinder with dip tube, 2: Pump, 3: CO_2 flowing valve, 4: Reaction cell, 5: Eluting valve, 6: collection vial)

Figure 3.4: Supercritical carbon dioxide reaction system photograph

(1: CO_2 cylinder, 2: Syringe pump, 3: temperature controller, 4: Pump controller, 5: Reactor)

The transesterification reactions were carried out in reaction cell. Compositions of the reaction mixtures were extracted fat (0.5 g), lipase (Novozym 435) and methanol with different quantities. After both substrates and lipase were added to the cell, CO_2 flowed from the CO_2 cylinder into the high pressure syringe pump. This high pressure pump pressurized the CO_2 to the desired pressure. 200 bar reaction was the recommended pressure found in previous work of Varma and Madras [120]. When both the desired pressure and temperature were reached, the reaction was started. This is done by opening the valve allocated between the pump and reaction cell. While the valve that elutes reaction products was sealed. As soon as the desired reaction time reached, reaction cell was depressurized by opening elution valve. Once the reaction product has been collected, it was dissolved in known *n*-hexane and conversion was determined using GC.

The variation of temperature in the system was controlled with ± 1°C and the variation of pressure was ± 1 bar. Effects of reaction temperature, different alcohol to fat molar ratios and enzyme loading on product yield were investigated. In addition, the recycling of Novozym 435 was also studied by the repeating use of the lipase with fresh substrates.

3.3 LAMB MEAT FAT PROFILE

Main fatty acid compositions of extracted fat were determined by direct transesterification of the fat to FAME according to the procedure described by Rule [188] with some modifications. About 10 mg of extracted fat was placed at the bottom of the tube and dissolved in 0.75 ml of chloroform-methanol prepared mixture (1:1, v:v). This was followed by adding 0.25 ml of 14% BF_3-methanol mixture. Mixture was heated in a water bath at (100°C) for 45 min and then cooled down followed by adding 1ml of distilled water (DW) and 2 ml of *n*-hexane, eddying for 1 min and centrifuging at a low speed. Upper phase was collected and *n*-hexane was evaporated while the residue was dissolved in 100µl of hexane and became ready for GC analysis.

3.4 FAME ANALYSIS

FAMEs amounts were determined using gas chromatograph (Chrompack CP-9001, Holland) fitted with a flame-ionization detector (FID) based on the retention times compared to that of external standards and equipped with Omega-wax 320 capillary column (30 m length× 0.32 mm internal diameter × film thickness of 0.25 µm; Supelco). The injector and detector temperatures were set at 250°C. Column temperature was programmed at 50°C during 2 min and increased to 100 °C at a rate of 20 °C min^{-1}, then increased to 240 °C at a rate of 10 °C min^{-1}, which was kept for 11.5 min. The injection volume was 0.5µL. Hydrogen and oxygen were supplied for the FID detector. FAME yield was calculated by the following Eq. (3.3).

$$\text{FAME yield } (\%) = \frac{\text{Weight of produced methyl ester}}{\text{Initial fat weight}} \times 100\% \qquad \text{Eq. (3.3)}$$

3.5 EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS

Response surface methodology (RSM) was applied to evaluate the effects of temperature, pressure and CO_2 Flow rate on extraction yield. A full 3^3 factorial design was selected to optimize the extraction operating conditions of fat extraction in SC-CO_2. The 27 experiments were performed and run duplicated and randomized. To determine possible interactions of process variables and their effect on the extraction yield, an analysis of variance (ANOVA) was carried out according to the Minitab 15 (MiniTab Inc,). The significance level was stated at 95%, with *p*-value 0.05.

CHAPTER FOUR: RESULTS AND DISCUSSION

CHAPTER FOUR: RESULTS AND DISCUSSION

In this study, the enzymatic transesterification of biodiesel from lamb meat fat and methanol using commercial immobilized lipase from *C. antarctica* in SC-CO_2 reaction medium has been studied. Influences of several reaction parameters, such as enzyme loading, reaction temperature and methanol molar ratio to fat were investigated. Additionally, effectiveness of SC-CO_2 extraction and yield of fat extracted from lamb meat has been investigated. Extraction yield and efficiency are affected by several parameters, such as particle size, moisture content of the feed, extraction temperature, pressure and solvent flow rate. The following discussion emphasizes the impact of these processing parameters on the yield of fat obtained from meat samples.

4.1 FEED SAMPLES CHARACTERIZATION

In supercritical extraction, similar to most other extraction methods, sample matrix characterization (e.g., moisture content, average sample particle size and fat content) plays a significant role in system efficiency to extract the desired compound. Therefore, feed sample characterization was studied to optimize system efficiency in extracting the fat from meat samples.

4.1.1 Water content

Various studies showed that the presence of water in a sample matrix affect fat extractability [189, 190]. Reducing the water content to a minimum value has become essential to obtain good results. Water forms a film over the fat and prevents SC-CO_2 reaching it. It can be reduced using several techniques including; spray drying, solar drying, osmotic dehydration, vacuum drying, freeze drying, microwave drying and drying using an agent. Among the possible techniques, vacuum and freeze drying were used in this work. Spray drying is not practical with the samples used in this work, meat. Solar, osmotic and microwave drying are also not suitable as they may affect final product

texture. Drying agents were also avoided, because the presence of the agent residue after separating from the sample may impede extraction process. Moreover, the residue of chemicals in the sample that will be used as food source is not acceptable.

The ability of the two elected techniques to remove water from samples was studied by determining the percentage of water that can be removed. Water removal was determined using Eq. (4.1).

$$\text{Relative weight loss}\,(\%) = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial sample weight}} \times 100\% \qquad \text{Eq. (4.1)}$$

With the three purchased samples (Australia leg, Australian shoulder and Indian leg), obtained results (Figure 4.1) showed that the effectiveness of both techniques were almost identical. However, vacuum drier was able to remove slightly more water from Australian samples than freeze drier. Vacuum drier was able to remove 68.8, 70.3 and 75.3% of the sample weight as water from Australia leg, Australian shoulder and Indian leg, respectively, whereas freeze drier was able to remove 65.3, 68.3 and 76.3%, respectively. Inspite of this, it was noticed that vacuum dried samples general appearance and texture of were affected which contrast with the objective of producing HLFM for human consumption, Therefore, freeze drying was selected as a preferred technique inspite of being expensive.

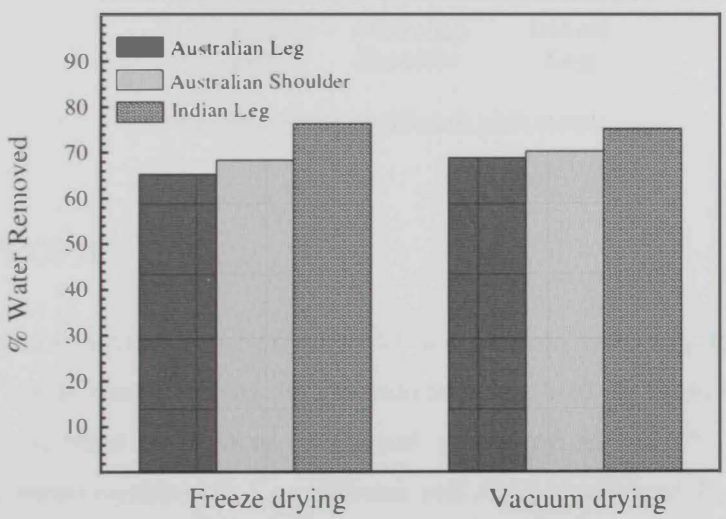

Figure 4.1: Relative weight losses from meat samples using vacuum and freeze driers

4.1.2 Fat content

Total amount of fat content in the samples was determined using soxhlet extraction system on the assumption that the soxhlet extraction can extract the whole amount of fat present in the sample. It was noticed that extracted fat was in solid state and white in colour whilst extracted sample became lighter after extraction. Solvent extraction results (Figure 4.2) showed that the two Australian samples contain almost the same amount of fat, but the Indian sample contains lowest amount of fat in comparison to the other two. Australian leg have the largest amount of 0.36 g-fat per gram of freeze dried sample , whereas Indian leg sample contains only 0.16 g-fat per gram of freeze dried sample.

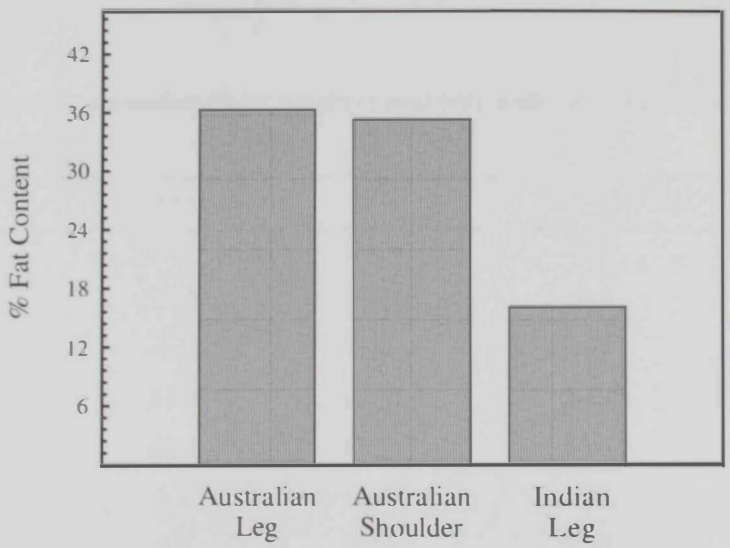

Figure 4.2: Fat content of different meat samples

4.2 FAT PROFILE

Fatty acid compositions of extracted fat at optimum conditions, 45°C, 500 bars and 3ml min^{-1}, were determined by direct transesterification of the fat to FAME where FAMEs were analyzed GC-FID as mentioned previously. GC results showed that extracted fat contains mystric acid, $C_{14:0}$, palmitic acid, $C_{16:0}$, stearic acid, $C_{18:0}$, oleic acid, $C_{18:1}$, and linoleic acid, $C_{18:2}$ and linolenic acid, $C_{18:3}$. Percentage composition by weight of total fatty acids is shown in Table 4.1. As can be seen, difference in total unsaturated

fatty acid and saturated fatty acids are insignificant, 52.1 and 47.9%, respectively. Mostly, oleic acid is the most common unsaturated fatty acid, 85.3% of total unsaturated fatty acid, whereas the main saturated acid is stearic acid, followed by palmitic acid. These results were similar to those with of Williams [169], Droulez et al. [191], Manso et al. [192] and Demirel et al. [193]. Droulez et al. [191] reported that in Australian lamb meat fats, the proportions of the two fatty acids are similar with little variation between cuts in the proposition of fatty acids, whereas, Williams [169] reported that in Australian red meat, saturated fatty acids content comprises, on average, 48% of meat fat. In this study, almost, more than 90 % of the fatty acid composition was composed of palmitic acid (19.2%), steric acid (26.3%) and oleic acid (44.43%). Figure 4.3 shows GC chromatogram of fat extracted from freeze dried sample.

Table 4.1: Fatty acid composition (% by weight of total fatty acids) of extracted fat using SC-CO_2 at optimum conditions.

Fatty acid	[191]	[192]	[193]	This study
$C_{14:0}$	2.6	2.64	3.6	2.36
$C_{16:0}$	19.5	23.75	20.4	19.22
$C_{16:1}$	1.5	1.41	1.97	--
$C_{18:0}$	14.3	16.39	20.2	26.32
$C_{18:1}$	43.1	42.31	36.4	44.43
$C_{18:2}$	6.7	6.40	4.65	2.42
$C_{18:3}$	2.5	0.44	2.27	4.30
$C_{20:4}$	2.0	4.04	1.26	--
$C_{20:5}$	0.8	0.51	1.23	--
$C_{22:5}$	1.2	0.80	0.77	0.95
$C_{22:6}$	0.4	0.42	0.38	--
SFA	36.4	42.78	44.2	47.9
MUFA	44.6	43.72	38.37	44.43
PUFA	13.6	12.61	10.56	7.67

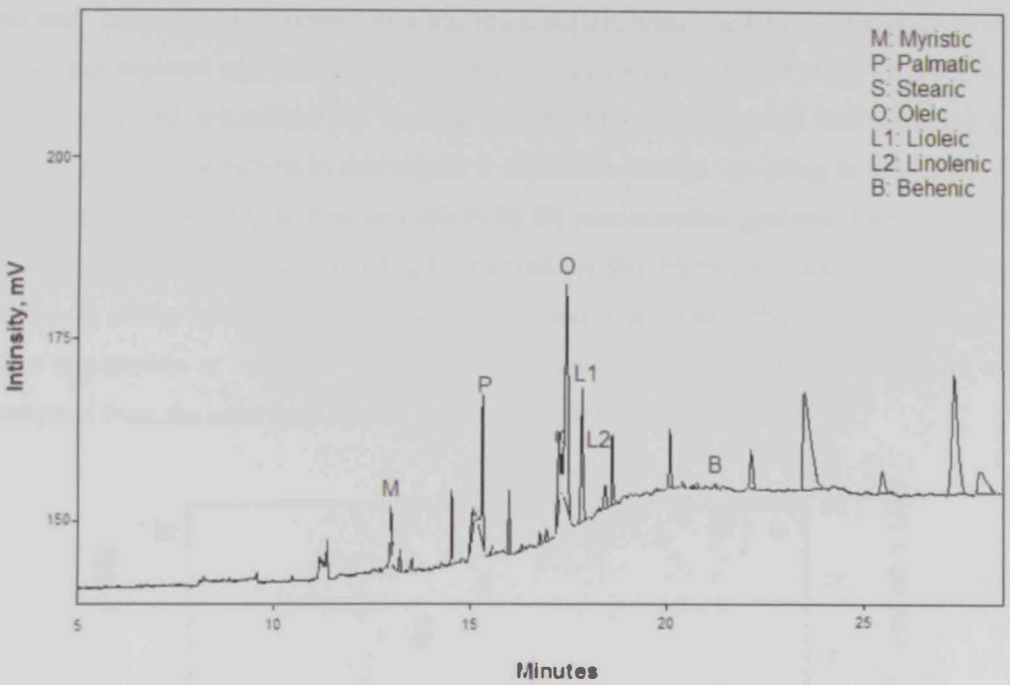

Figure 4.3: GC chromatogram of fat extracted from freeze dried sample

4.3 SC-CO_2 EXTRACTION TIME COURSE

To study extraction time course, extraction yield as a function of extraction time was plotted. Figure 4.4 shows SC-CO_2 extraction curve for a fat extracted from Australian shoulder samples at 45°C, 300 bars and 3 ml min^{-1}. Represented data are of average value of duplicate runs. Extraction yield was defined as weight of extracted fat divided by initial sample weight whereas extraction efficiency was defined as SC-CO_2 extraction obtained yield, divided by total sample fat content which was determined by soxhlet extraction system using *n*-hexane solvent.

At the beginning of extraction, there was a linear dependence between extracted fat and passed CO_2 and the rate of extraction was almost constant. In this region of extraction, sample particles are totally covered by the CO_2 and extraction rate depends on the fat solubility in SC-CO_2. When SC-CO_2 came in contact with the sample, the fat on the surface was rapidly solubilised and extracted where extraction rate is limited by the solubility. In this extraction region about 74.3 % of total fat present in the sample was

obtained. This region is followed by a transition region, when the fat on the surface of the particles is depleted and particles are no longer coated with the fat. SC-CO_2 diffused into the particles and solubilised the internal fat and then SC-CO_2 with the extracted fat diffuses out. The extraction in this region is diffusion- limited, resulting in a decrease in extraction rate. This step is slow and driven by fat concentration gradient. Finally, a state where there is no more fat extracted is reached. In this region, extraction rate is slow movement ability of the fat within the sample matrix become difficult, thus, extraction curve approaches a constant value. The reproducibility of experimental results are confirmed from the error bars, shown in the figure, which were less than 1%.

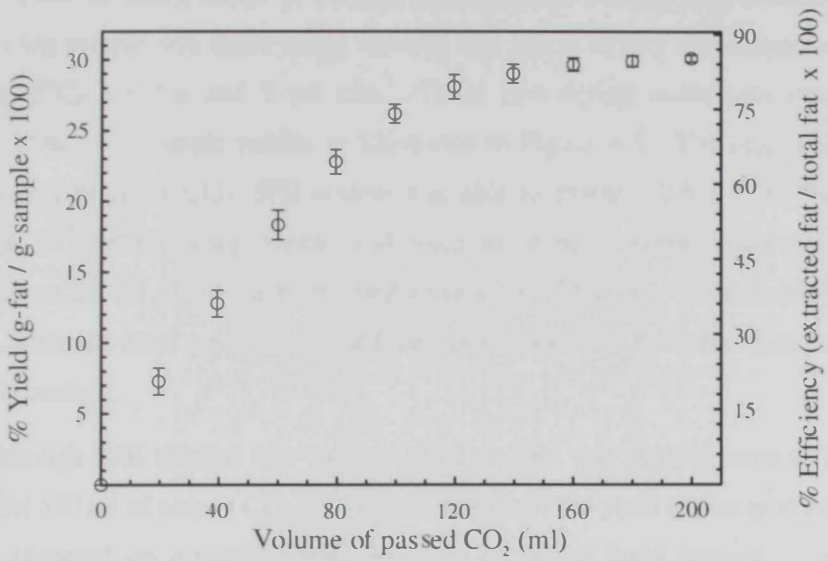

Figure 4.4: Overall SC-CO_2 extraction curve

As can be seen, after passing 200 ml of SC-CO_2, more than 80% of initial fat content were extracted in only 70 min whereas in soxhlet extraction 250 ml of *n*-hexane was consumed and took about 6 hours to extract all present fat. Moreover, soxhlet extraction required to heat up extracted sample to remove residual solvent, and as mentioned earlier, the use of chemicals are not recommended for food products, like in our case. Therefore, SC-CO_2 extraction was preferable.

4.4 SAMPLE PRE-TREATMENT EFFECT

Samples pre-treatment consist of minimizing effect of sample water content and mechanical grinding to decrease the particle size. For preliminary tests, the effects of fat content, water content and mechanical grinding on extraction yield fat and efficiency were determined.

4.4.1 Water content

In order to study affect of sample water content on SC-CO_2 extraction yield, Australian leg sample was dried using vacuum and freeze drying and subjected to SFE system at 45°C, 300 bar and 3 ml min^{-1}. These two drying techniques results were compared with fresh sample results as illustrated in Figure 4.5. The results show that after passing 200 ml of CO_2, SFE system was able to extract 72.6 and 85.7% of total amount of fat present using freeze and vacuum dried samples, respectively. This corresponds to 30.4% of the vacuum dried sample and 25.76% of freeze dried sample. However, when un-dried sample was used the system was able to extract less than 2% of the total fat content.

Although SFE showed that vacuum dried sample was slightly more efficient. Its yield in first 120 ml of passed CO_2 was almost similar to the yield of freeze dried sample. This was expected since vacuum drier was able to extract large amount of water that present inside the matrix, as shown in Figure 4.1. Therefore SC-CO_2 can reach the fat more easily. High water content forms a film around fat particle, act as a mass transfer barrier and inhibits the contact between SC-CO_2 and the fat and prevents CO_2 from contacting and hence extracting the fat. This results agrees with the previous works done by King et al. [184] who showed that fats were more effectively extracted from dried meat samples using SC-CO_2.

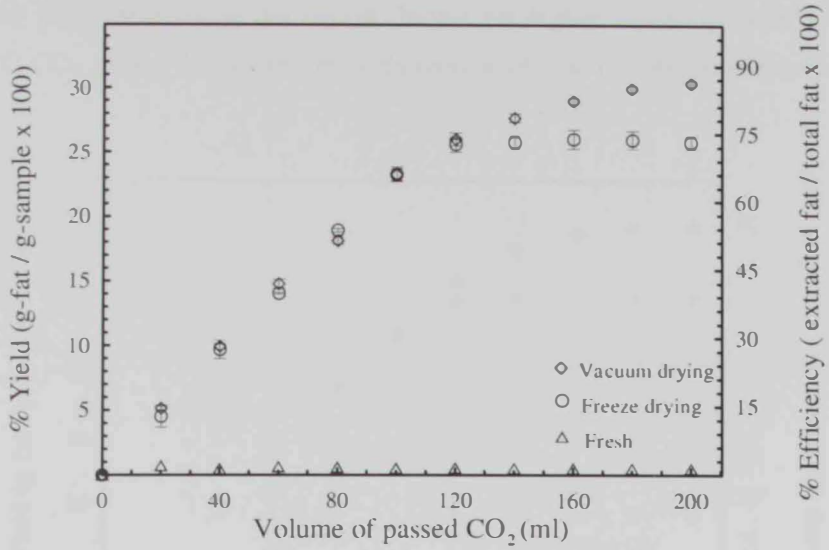

Figure 4.5: Effect of different drying techniques on extraction yield

4.4.2 Fat content

To determine the effect of initial fat content on extraction yield and SFE system efficiency, extraction experiments with samples from different sources were compared. Figure 4.6 shows the three different freeze dried samples extraction yield and system efficiency curves at 45°C, 300 bars and 3ml min^{-1}. SC-CO_2 results showed good agreement with solvent extraction results (section 4.1.2) which showed that Australian samples contain higher amount of fat than Indian one. SC-CO_2 was able to extract 25.8, 31.4 and 9.1 % fat from Australian leg, Australian shoulder and Indian Leg, respectively. This corresponds 72.7, 88.6 and 25.7% extraction efficiency respectively.

In addition, as shown in the Figure 4.6, for the first 20 ml of passed SC-CO_2, SC-CO_2 was able to yield 4.5% fat from Indian leg meat and further increase of passed SC-CO_2 increased slightly to reach maximum yield of 9.1% after passing 200 ml of SC-CO_2. Inspite that freeze dryer removed 75% of sample weigh as water, amount of water remained in the dried sample might be still high in comparison with sample fat content which resulted low system efficiency in extracting the fat. On the other hand, in Australian samples, in first 100ml of passed SC-CO_2, 23% of initial sample weight was extracted. Further increase in SC-CO_2 increased fat extraction to yield 25.8 and 31.4%, respectively. Deviation of Australian leg yield from Australian shoulder results might be due to amount of water that present in the sample and ability of SC-CO_2 to penetrate and

extract the fat present inside the matrix. Indian fat higher solubility in first 20 ml of passed SC-CO_2 might due to the easy extraction of the fat presence near to sample surface.

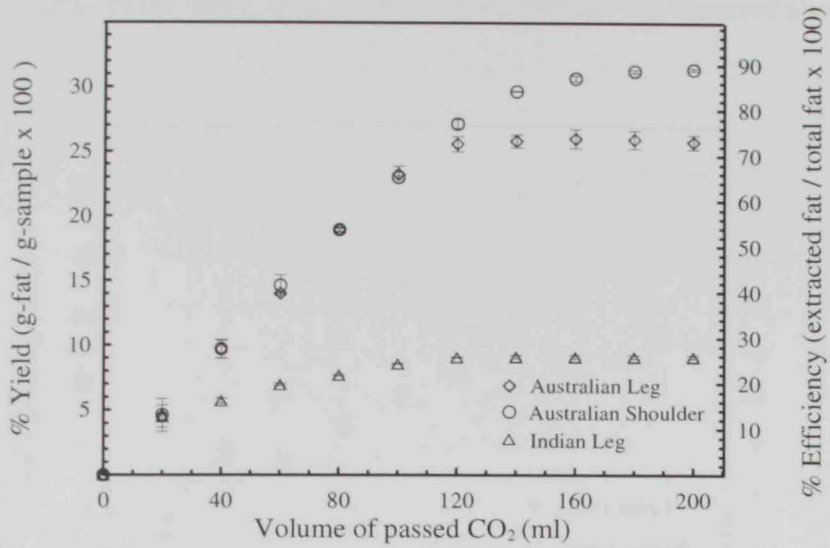

Figure 4.6: Effect of initial fat content on the extraction yield and system efficiency

4.4.3 Mechanical grinding

As a result of the low solubility of some compound, it was suggested to reduce sample particles size to reduce extraction time by many investigators [190, 194]. Therefore, breaking the sample down to small particles leads to expose soluble fats that present in the solid matrix to the surface and hander them easily reachable by the solvent. Particle size effect has been investigated in many studies by grinding the sample and fractionating ground samples into different sizes using sieves [190, 194]. In this study, ground samples were used without any fractionation. An Australian shoulder sample was used to study the effect of this pre-treatment step on extraction yield where ground samples results were compared to un-ground samples. Samples grinding were performed using mechanical grinder (Molinex) and fat was extracted at 45°C, 300 bars and 3ml min^{-1}.

Figure 4.7 shows the effect of grinding on extraction yield and SFE efficiency. It was noticed that grinding improved extraction yield. This is attributed to the fact that

particle size controls CO_2 diffusion through sample matrix. By increasing surface area and reducing diffusion path which lead to increase mass transfer of the dissolved fat. However, SC-CO_2 was successful to extract fat from un-ground meat which is promising for using chunks in the future. This extraction enhancement was observed also by [190, 195].

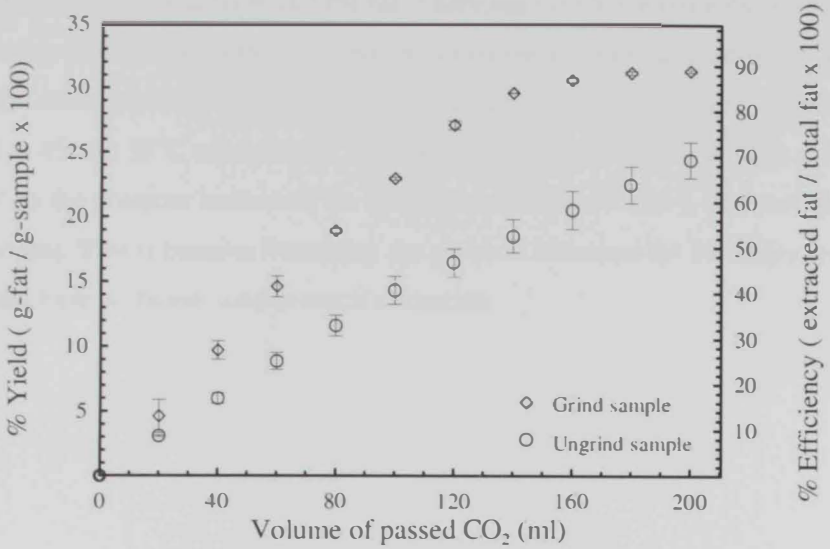

Figure 4.7: Effect of sample particle size on extraction yield and system efficiency

4.5 EFFECT OF PROCESS PARAMETERS

For the study of process parameters effect, three different pressures, 300, 400 and 500 bar, three different temperatures, 35, 45 and 55°C and three different flow rates, 3, 4 and 5 ml min^{-1} were employed with freeze dried, ground Australian shoulder sample. Extraction yield was plotted verses the amount of SC-CO_2 passed through the extractor.

4.5.1 SC-CO_2 flow rate

To investigate the effect of carbon dioxide flow rate on extraction yield ad SC-CO_2 extraction system efficiency, a set of experiments was carried out at different operating conditions with CO_2 flow rate varying from 3 to 5 ml min^{-1} using 2.5g of ground freeze dried Australian shoulder meat samples.

Figure 4.8(a-c) shows effect of SC-CO_2 flow rate at 35°C on extracted fat accumulation yield and system efficiency. As shown, extraction yield decreased as CO_2 flow rate increased, for example, at 300 bars, after passing 200 ml of CO_2, maximum extraction yield of 27.8, 25.5 and 23.5% were obtained with 3, 4 and 5 ml of passed SC-CO_2, respectively. This is due to the decrease of residence time that results in shorter contact time between the solvent and the fat. Therefore the CO_2 leaving the extraction cell may not being saturated with fat. This indicates that the intra-particle diffusion resistance controls the extraction. This trend was also observed at other pressures. Similar results are found at 45 and 55°C as shown in Appendix A (Figures A.1 and A.2). It can also be noted that as the pressure increases, the difference between 3 and 4 ml min^{-1} flow rates tends to reduce. This is because increasing the pressure increases the solubility, and hence both 3 and 4 have sufficient time to reach saturation.

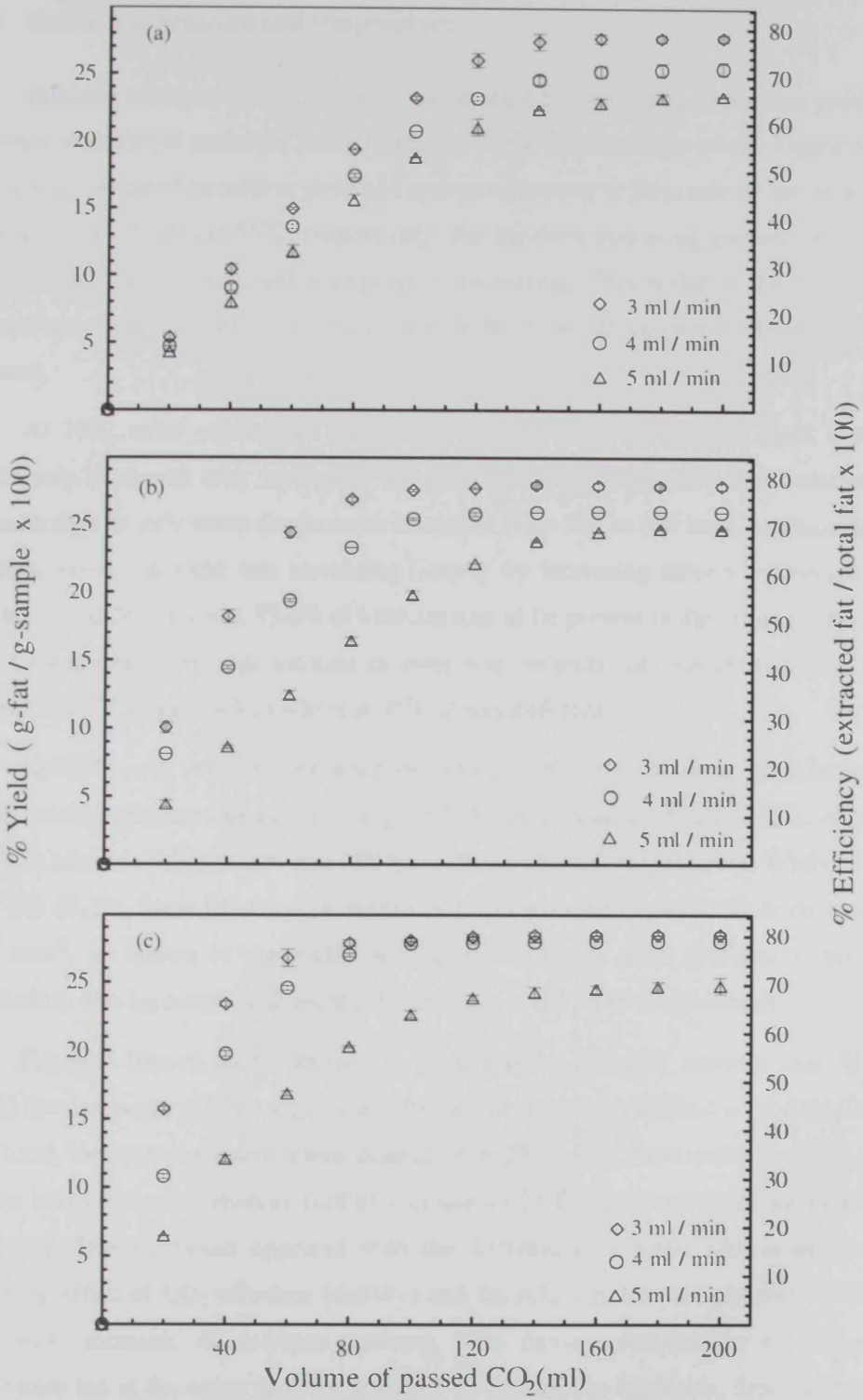

Figure 4.8: Effect SC-CO_2 flow rate at 35°C and different pressures on extraction yield and efficiency (a) 300 bar, (b) 400 bar and (c) 500 bar

4.5.2 Extraction pressure and temperature

Pressure effect on extraction yield was studied by comparing extraction yield and efficiency at different pressures while fixing the temperature and flow rate. Figure 4.9(a-c) shows the obtained extraction yield and system efficiency at flow rate of 3ml min^{-1} and temperature of 35, 45 and 55°C, respectively. For the three operating temperatures, yield and system efficiency increased with pressure increasing. This is due to the fact that as pressure increases, CO_2 density increases, and therefore, solvent power to dissolve that fat increases.

At 35°C, after passing 80 ml of CO_2 to the extraction cell, fat yield was not significantly increased with increasing the pressure from 400 to 500 bars, whereas, it increased significantly when the pressure increased from 300 to 400 bars. Additionally, at 300 bars, extraction yield was increasing linearly by increasing amount of passed CO_2 reaching round 26.0% yield, 73.6% of total amount of fat present in the sample, when 120 ml of CO_2 passed. After that increase in yield was insignificant. Similar behaviour was obtained at 55°C (Figure 4.9.c) whilst at 45°C it was different.

At 45°C, with the three operating pressures, as mentioned before, yield increased with increasing pressure. As shown in Figure 4.9b, after passing 140 ml of CO_2, obtained yield was almost similar at 400 and 500 bars, 29.3 and 30.9, respectively. Whilst at 300 bar it was 29.2%. Same behaviour was obtained also with the two other flow rates, 4 and 5 ml min^{-1}, as shown in Appendix A (Figures A.3 and A.4). Extraction rate was significantly also increased with increasing pressure at the three temperatures.

Figure 4.10(a-c) shows extraction yield for the different temperatures. It was noticed that temperature has a significant effects on extraction yield and solubility. On the other hand, increasing extraction temperature from 35 to 45°C increased extraction yield and the extraction rate whereas further increase to 55°C decreased both the yield and initial rate. This behaviour appeared with the different flow rates. This is due to the competing effect of CO_2 salvation (density) and fat volatility (vapour pressure) with the temperature increase. At constant pressure, CO_2 density decreases with increasing temperature but at the same time the volatility of the fat also increases. Since increasing temperature from 35 to 45°C increased extraction yield and extraction rate, volatility effect was the dominant, whereas further increase in temperature, density effect became

the dominant, as shown in Figure 4.10(a-c). Similar trend was observed by Liza et al [196] when they extracted bioactive flavonoid from peach kaca. Figures A.5 and A.6 in Appendix A shows obtained results at 4 and 5 ml min^{-1}, respectively.

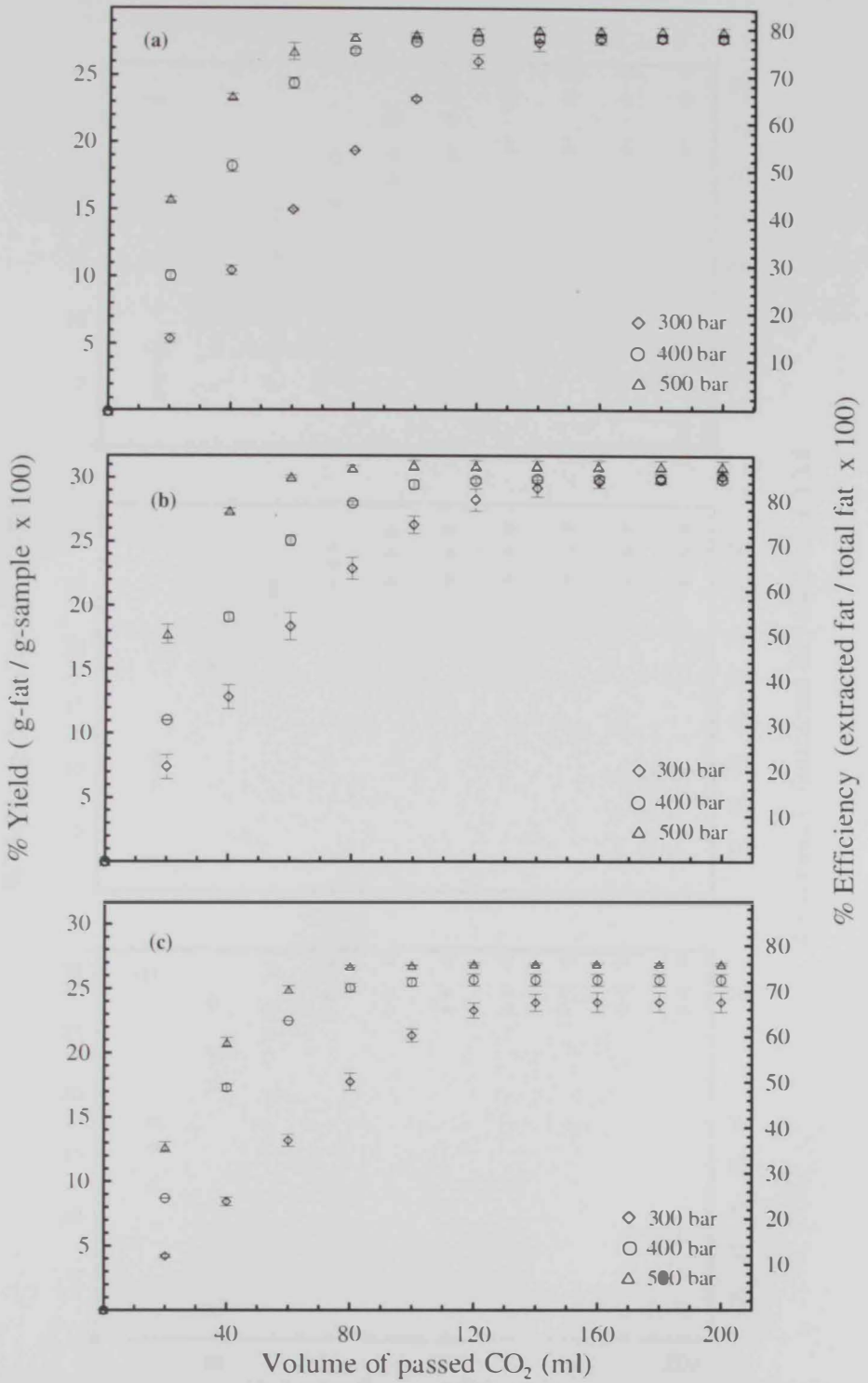

Figure 4.9: Effect of SC-CO_2 pressure at 4 ml min^{-1} different temperatures on extraction yield and efficiency (a) 35°C, (b) 45°C and (c) 55°C

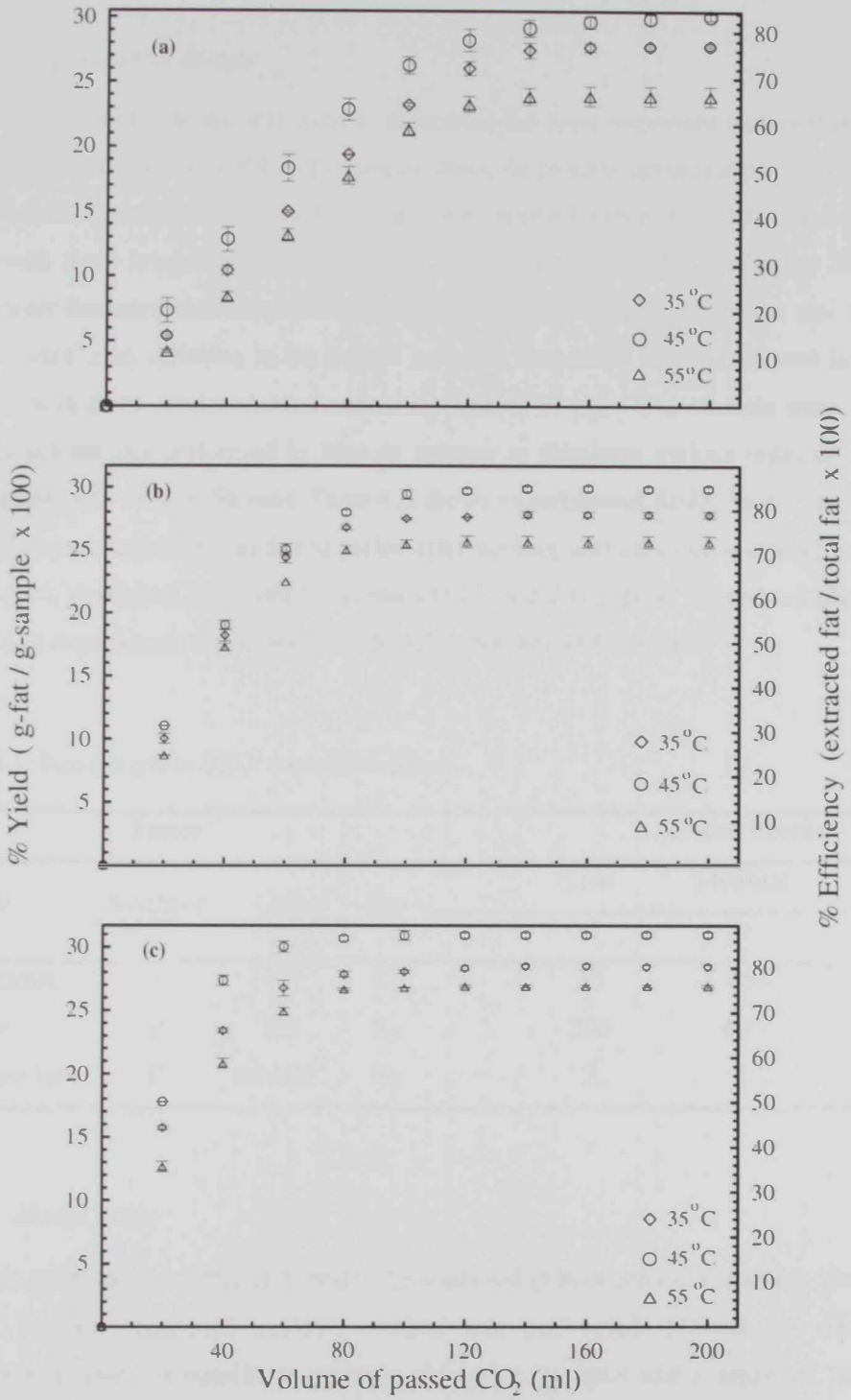

Figure 4.10: Effect of SC-CO_2 temperature at 3 ml min^{-1} and different pressures on extraction yield and efficiency (a) 300 bar, (b) 400 bar and (c) 500 bar

4.5.3 Process optimization

4.5.3.1 Experimental design

Experimental design was used to determine the most important factors that might affect SC-CO_2 extraction yield and optimize them. In process optimization, effect of the three variables on SC-CO_2 extraction yield was studied using 3^3 full factorial design (FFD) with three levels for each factor (low (-1), medium (0) and high (1)). Selected factors were the extraction temperature, extraction pressure and SC-CO_2 flow rate. Coded and un-coded used variables in the design and their respective levels are listed in Table 4.2. FFD with three level and three variables required 27 runs. Experiments were carried out in duplicate and performed in random manner to eliminate various types of biases. This resulted in a total of 54 runs. Table 4.3 shows experimental design matrix as well as obtained experimental and predicted yields after passing 200 ml of CO_2 in each run. As can be seen, maximum observed fat yields (31.20 and 30.62 g-fat/ g feed-sample) were obtained in experiments that correspond to 45°C, 500 bar and 3 ml min^{-1}.

Table 4.2: Factor levels in full 3^3 factorial analyses.

Factor				Coded Levels		
Variable	Symbols	Unit	Key	Low	Medium	High
				-1	0	1
Temperature	T	°C	X_1	35	45	55
Pressure	P	bar	X_2	300	400	500
CO_2 flow rate	F	ml min^{-1}	X_3	3	4	5

4.5.3.2 Model fitting

In order to investigate and model the relationship between the extraction yield and selected factors, regression analysis method was performed. Minitab 15 statistical software was used for non-linear multiple regression analysis and analysis of variance (ANOVA). Second order polynomial regression model was tested to express the yield as a function of the three selected variables as shown in Eq. (4.2), where Y represents the

yield response, X_i and X_j are the levels of the variables, β_0 is a constant, β_i, β_{ii} and β_{ij} are linear, quadratic and interactive coefficients, respectively.

$$Y = \beta_o + \sum \beta_i X_i + \sum \beta_{ii} X_{ii}^2 + \sum \sum \beta_{ij} X_{ij} \qquad \text{Eq. (4.2)}$$

Table 4.3: Experimental design conditions and experimental and predicted yield data.

Run Number	Process variables			Yield (g-fat / g-sample)	
	Temperature (°C)	Pressure (bar)	Flow rate (ml min^{-1})	Experimental	Predicted
1	45	300	5	25.49	28.01
2	55	500	3	26.82	28.19
3	55	400	5	24.59	26.13
4	35	400	3	27.66	30.37
5	35	500	4	27.95	27.34
6	35	400	5	24.59	24.31
7	35	500	5	24.30	24.31
8	45	400	5	26.59	28.01
9	45	400	4	28.31	30.04
10	45	500	4	29.48	30.04
11	45	500	5	27.21	28.01
12	55	500	5	25.50	26.13
13	45	300	5	25.79	28.01
14	55	300	5	25.49	26.13
15	45	500	3	30.62	32.07
16	55	500	5	25.21	26.13
17	45	500	3	31.20	32.07
18	35	300	4	25.15	27.34
19	35	300	4	25.86	27.34
20	45	300	3	29.94	32.07
21	45	400	4	27.90	30.04
22	45	300	4	27.95	30.04
23	35	500	3	28.25	30.37

24	45	500	4	29.30	30.04
25	55	400	3	25.97	28.19
26	55	400	3	25.33	28.19
27	45	400	3	29.70	32.07
28	55	300	3	24.45	28.19
29	35	500	3	28.58	30.37
30	35	500	4	27.94	27.34
31	35	300	5	22.10	24.31
32	35	400	3	28.00	30.37
33	45	500	5	27.20	28.01
34	55	500	4	24.70	27.16
35	35	300	5	22.71	24.31
36	45	300	4	27.40	30.04
37	35	400	5	24.71	24.31
38	55	300	5	25.79	26.13
39	55	400	4	25.66	27.16
40	45	300	3	30.28	32.07
41	55	300	4	23.48	27.16
42	55	500	3	26.98	28.19
43	35	300	3	27.59	30.37
44	35	400	4	25.91	27.34
45	55	400	4	24.94	27.16
46	35	400	4	25.90	27.34
47	35	500	5	25.14	24.31
48	55	500	4	25.57	27.16
49	45	400	5	27.72	28.01
50	35	300	3	27.97	30.37
51	55	400	5	25.82	26.13
52	55	300	4	23.69	27.16
53	55	300	3	23.37	28.19
54	45	400	3	30.03	32.07

Degree of factor significant is represented by *p*-value. When variables and their interaction have a *p*-value smaller than 0.05, it influences extraction yield in a significant way. On the other hand, if it has *p*-value smaller than 0.001, it influences in extraction yield high significant way.

ANOVA analysis for second order polynomial model of the studied factors obtained a *p*-value of 0.000 (*p*-value < 0.05), indicating that at least one of the regression coefficient is different than zero. Multiple second order polynomial regression coefficients were obtained by employing a least squares technique. Table 4.4 shows obtained results from Minitab 15 statistical software. For the fat yield, examination of these coefficients indicated that linear and quadratic terms of temperature, pressure and extraction time were highly significant (*p*-value < 0.01). From the statistical analysis of regression coefficients, extraction yield was highly affected by temperature (*p*-value < 0.001) whereas it was significant affected by the flow rate (0.001 < *p*-value < 0.05). Moreover, there was a significant interaction between the temperature and flow rate. Based on obtained *p*-value and ignoring the insignificant terms, the following second order model was obtained:

$$Y = 2.10\,T - 6.52\,F - 0.0279\,T^2 + 0.0999\,T\,F \qquad \text{Eq. (4.3)}$$

Predicted values yield was determined using the regression model and compared with the experimental values. Large value of determination coefficient (R^2 =0.89) shows that the model sufficiently represents the experimental results. The linear, quadratic and interaction coefficients in the second order polynomial model were used to generate a three-dimentional response surface graph.

Table 4.4: Regression coefficient, standard error and *p*-value of the fitted second order polynomial model.

Coefficient	Value	Standard error	*p*-value
β_0	-7.683	8.031	0.334
Linear			

β_T	2.1049	0.2178	0.000
β_P	0.01956	0.02005	0.334
β_F	-6.525	2.005	0.002
Quadratic			
β_T^2	-0.027931	0.002208	0.000
β_P^2	-0.00000613	0.0002208	0.783
β_F^2	0.1259	0.2208	0.571
Interaction			
β_{TP}	-0.0000945	0.0001561	0.548
β_{TF}	0.09990	0.01561	0.000
β_{PF}	-0.000696	0.001561	0.658
R^2	0.89		

4.5.3.3 Response surface analysis

Response surface was developed using the reduced form of the fitted polynomial model (Eq. (4.3)). This was illustrated in a three-dimensional plot (3D) by plotting the yield, response, as a function of significant variables ,temperature and flow rate. Figure 4.11 represents the response surface plot of the extraction yield as a function of extraction temperature and pressure at the fixed pressure of 500 bar and fixed passed 200 ml of SC-CO_2. Pressure effect was ignored based on obtained results in previous section. As shown in Figure 4.11, at low temperature extraction yield increased with the rise of temperature. This is most possible due to the increased mass transfer rate. However, at higher temperature extraction yield decreased with the rise of temperature which most likely due to the decrease in SC-CO_2 density. SC-CO_2 has a positive linear effect which is mostly due to the decrease in resident time and contact between SC-CO_2 and the extractable fat. There was significant interaction between temperature and flow rate. At low temperature, it consumed more SC-CO_2 to reach steady state. However, at high temperature it consumed less SC-CO_2 to reach the steady state.

Optimum values of tested variables were obtained by deriving and solving Eq. (4.3) with respect to temperature. Flow rate variable was eliminated as a result of linear dependent between yield response and flow rate factor. Eq. (4.4) shows the differentiated

yield model. Differentiated equation predicted that 43°C is the optimum modelled temperature. Pressure of 300 bar and flow rate of 3 ml min^{-1} was selected as best conditions. This lead a maximum predicted extraction percentage yield of 32.04%.

$$\frac{dY}{dT} = 2.10 - 0.0558\,T + 0.0999\,F = 0.0 \qquad \text{Eq. (4.4)}$$

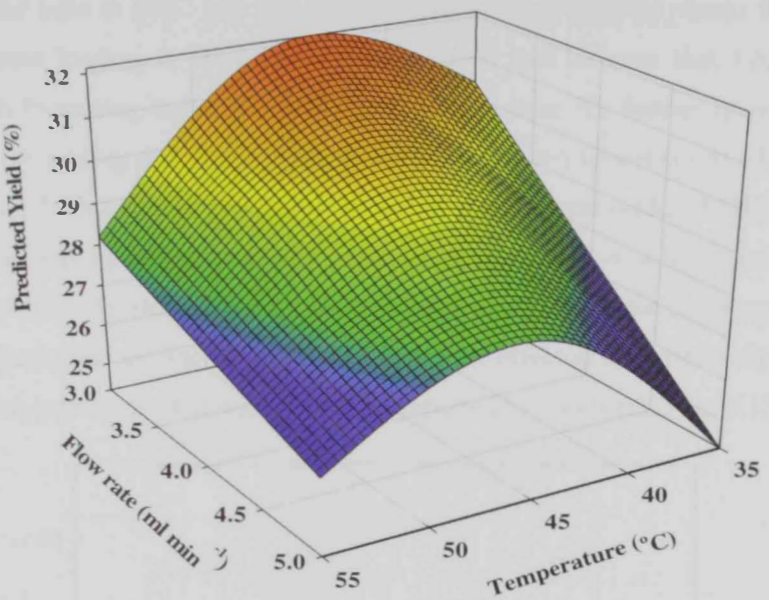

Figure 4.11: Fat yield surface plot as a function of temperature and flow rate

4.6 ENZYMATIC TRANSESTERIFICATION IN SC-CO_2

For enzymatic biodiesel production optimization using lipases, factors affecting FAME conversion have to be optimized. Studies showed that reaction temperature, methanol to oil ratio and enzyme loading are the most important factors. FAME yield was defined as amount of produced FAME per amount of fat used. Produced amount of

FAME was obtained from GC analysis. Figures B.1 - B.5 in Appendix B show GC chromatograms for different transesterification reactions conditions.

4.6.1 Effect of enzyme loading

In order to study the effect of enzyme quantity on biodiesel yield, Novozym 435 loading was varied in the range of 10% to 50%, with 20% interval (as a ratio to initial amount of fat extracted from lamb meat). Beside the amount of enzyme loading, all other variables were kept constant. The experiments were performed with 0.5 g fat, 4:1 methanol molar ratio at 50°C and 200 bars. FAME production yield versus time using different enzyme loading is shown in Figure 4.12. It can be seen that FAME yield increased with increasing enzyme loading and reaction time. To further investigate the effect of enzyme loading on FAME yield, the yield was drawn versus enzyme loading, as shown in Figure 4.13. It can be seen initially, at 5 hours, the increase in FAME yield with enzyme loading was linear. However, the effect start deviate from a linear behaviour at higher times and this deviation increases as time increases. This is due to the approaching of the equilibrium yield as time increases, and hence the effect of enzyme loading tends to diminish, which is in agreement with the results reported by Nouureddini et al [25].

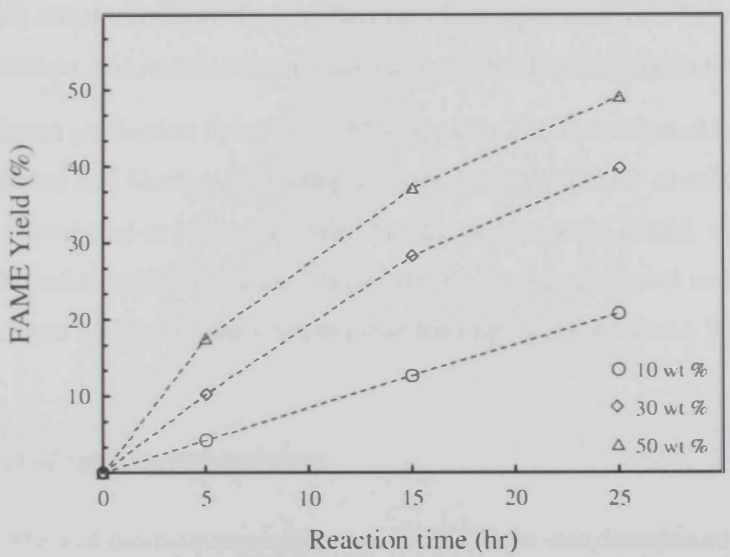

Figure 4.12: FAME production yield versus time using different enzyme loading

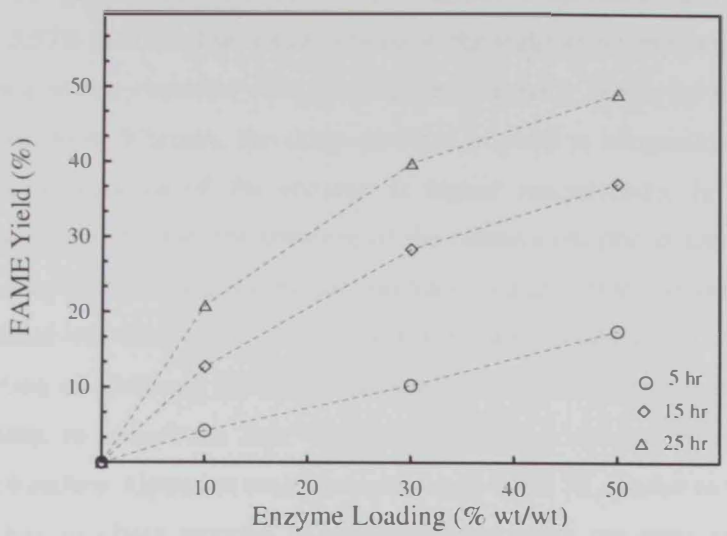

Figure 4.13: Effect of enzyme loading on FAME yield

Although the rate of reaction increased with increasing the enzyme loading, from an economical point of view and due to the high cost of the enzyme, the loading cannot be increased indefinitely. From the results shown in Figure 4.13 at 25 hrs, it can be seen that the yield increased significantly as the enzyme loading increased from 10% to 30%. However, this effect of enzyme loading becomes less significant as it increased from 30% to 50%. Therefore, it is not recommended to use enzyme loading higher than 30%.

Biodiesel production from oil in SC-CO_2 was also investigated by Madras et al. [29] and Rathore and Madras [51] using enzyme loading of 30% at 45°C, 8 hr reaction time and 5:1 methanol to oil molar ratio. Similar results were found, where as enzyme loading increased a sudden increase in ester formation was observed and the increase in the yield and was not significant when enzyme loading increased above 30% loading.

4.6.2 Effect of reaction temperature

The effect of reaction temperature on FAME yield was determined by carrying out enzymatic reaction in SC-CO_2 media at different temperatures in the range of 35°C to 60°C at 200 bars, 4:1 alcohol to fat molar ratio, 30% enzyme loading and 25 hr reaction time. As shown in Figure 4.14, increasing the temperature from 35°C to 50°C resulted in

increasing the yield from 8.3% 39.8%. At reaction temperatures above 50°C, FAME dropped to 5.57% at 60°C. The initial increase in the yield with temperature is mainly due to an increase in rate constants with temperature, and partly due to the reduction in mass transfer limitations. Whereas, the sharp decrease in yield at temperature above 50°C is due to the denaturation of the enzyme at higher temperatures. In addition to the deactivation of the enzyme, the presence of the inactive enzyme at the interface blocks the active enzyme from penetrating the interface, which further decreases the reaction rate. This trend was observed in all studies that investigated the effect of temperature on the production of biodiesel by lipase, however, the critical temperature at which the enzyme starts to deactivate was different, depending on the type of lipase and immobilized surface. Optimum reaction temperature of 50 °C, similar to the one found in this work, has also been reported in previous works using the same enzyme [197] for refined cotton seed transesterification to FAME in solvent free media.

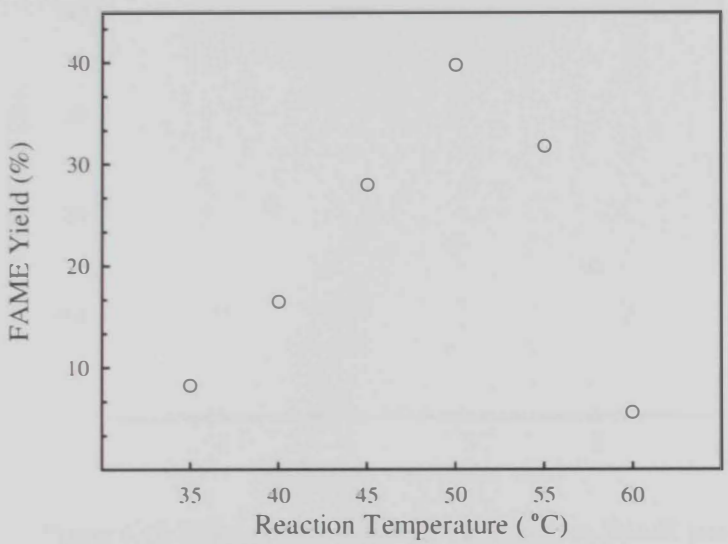

Figure 4.14: Effect of reaction temperature on FAME yield

4.6.3 Effect of methanol to fat molar ratio

The molar ratio of methanol to fat is one of the most important variables affecting the yields of FAME. Stoichiometrically, to produces FAME three molecules of methanol are required to react with one molecule of extracted fat. Increasing molar ratio should

increase forward reaction to produce more FAMEs, therefore, the effect of the molar ratio of methanol to fat was studied in the range between 3 and 6 on the yield of FAME formed and taking the average molecular weight of fat is 850 g mol^{-1}. Figure 4.15 shows obtained yield for different methanol to oil ratios using 30% enzyme loading at 50°C, 200 bar and for 25 hr reaction time. As expected, increasing methanol to fat ratio from stoichiometric resulted in increased FAME yield to an optimum value then the yield dropped. Highest FAME yield was obtained at 4:1 methanol to oil ratio. An increase of the molar ratio from 3:1 to 4:1 increased FAME yield more than three time yielding 39.9%. This finding is in a close agreement with previous results found in literature using the same enzyme and alcohol [197]. The drop after 4:1 ratio is due to inhibition of the lipase by the contact with insoluble methanol which exists as drops in the fat. Shimada et al. [198] reported that methanol solubility in triglyceride is $^1/_3$ of the stoichiometric amount.

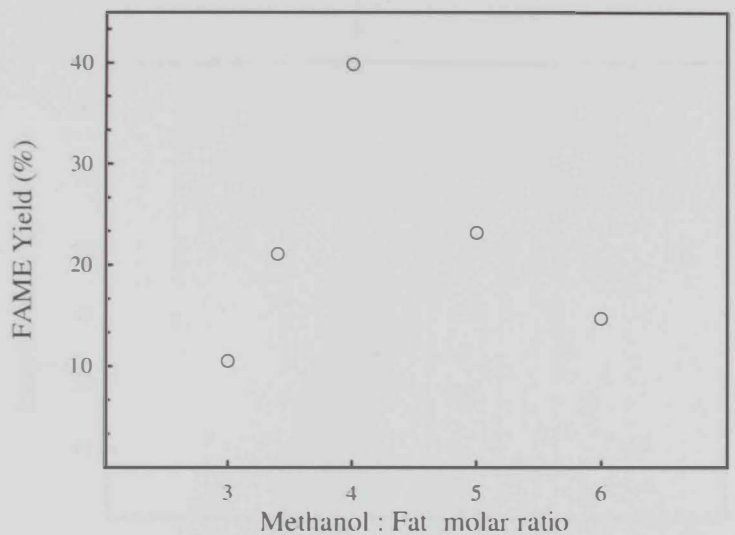

Figure 4.15: Effect of methanol to fat molar ratio on FAME yield

4.6.4 Enzyme reusability

From economical point of view, repeating use of immobilized enzyme makes the process more viable and reduces product cost. To study Novozym 435 reusability, at the end of each reaction, enzyme was filtered from the reaction mixtures; distilled water was used to wash the enzyme before introducing again for the next batch. The following batch

composed of reused Novozym 435 and fresh batch of substrates. This was repeated several times to examine the degree of enzyme stability. Experiments were performed at 50°C, 200 bar, 30% enzyme loading and 4:1 molar ratio for 25 hr. To determine relative Novozym 435 activity, first batch fat conversion was considered as 100 and conversions in followed cycles were expressed as relative conversions. Figure 4.16 shows enzyme relative activity for 7 cycles. It was noticed that relative Novozym 435 activity decreased significantly with the increasing number of batches. As illustrated in the figure, in the first recycle use, approximately 72% activity was obtained while after 7 cycles, it lost round 85% of its initial activity. The activity reduction may be due to enzyme loss during filtration step, since no additional quantities of enzyme were added. Similar behaviour was found by Iso et al. [35] where enzyme activity was decreased to about two-third, when it was used for second time. This effect will be further investigated using different washing solvents.

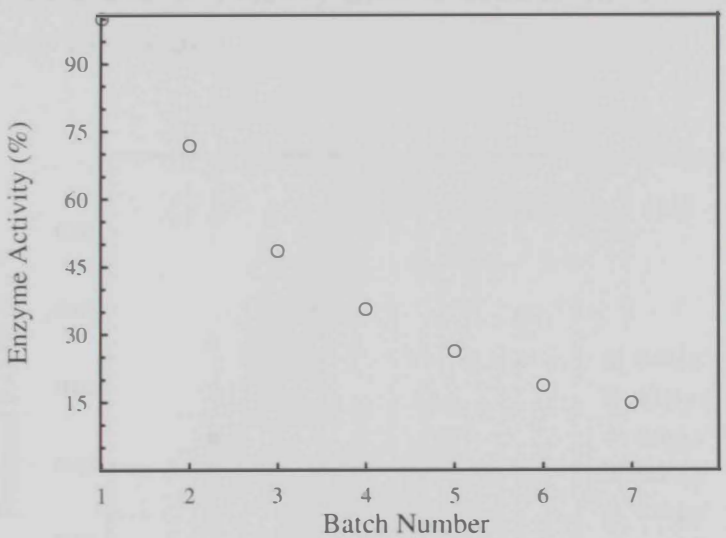

Figure 4.16: Change in Novozym 435 activity with the repeating use

4.7 ENZYME KINETIC STUDY

Biodiesel enzymatic reaction kinetic was studied using SC-CO_2 as a reaction medium and Novozym 435 lipase as a biocatalyst. The effect of changing methanol concentration on initial transesterification rate was studied at 50°C, 200 bar, 10 wt%

loading and 4 hr reaction. Five hours reactions and 10% enzyme loading were considered to investigate initial rate changes. Methanol concentrations were based on initial amount per initial fat used. Methanol initial concentration was termed as $g_{methanol}$ / g_{fat} whereas produced FAME concentration was termed as g_{FAME}/ g_{fat}. Since initial fat concentration was fixed, amount of required lipase was kept constant at 0.05 g.

In order to determine kinetic parameters, graphical demonstration of the initial reaction rates against methanol concentration at fixed fat concentrations was used. Initial rates were determined from FAME production yield verses time for different methanol concentrations as shown in Figures 4.17 and 4.18. Methanol concentration was varied from 0.12 to 3.2 g g^{-1} at fixed fat concentration. For the first two hours of reaction, increasing methanol mass concentration from 0.12 to 0.16 gg^{-1} increased produced FAME yield and initial reaction rate. FAME yield increased from 0.026 to 0.046 g g^{-1}. Further increase of methanol concentration above 0.16 reduced the yield and initial reaction rate. This trend agreed with the inhibitory effect of methanol on the enzyme obtained in section 4.5.3 for 25 hr reaction.

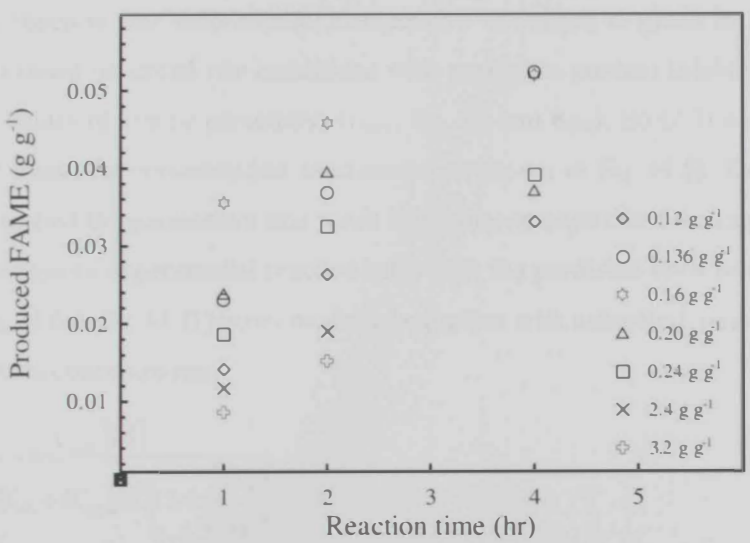

Figure 4.17: FAME production time course at different initial concentration of methanol

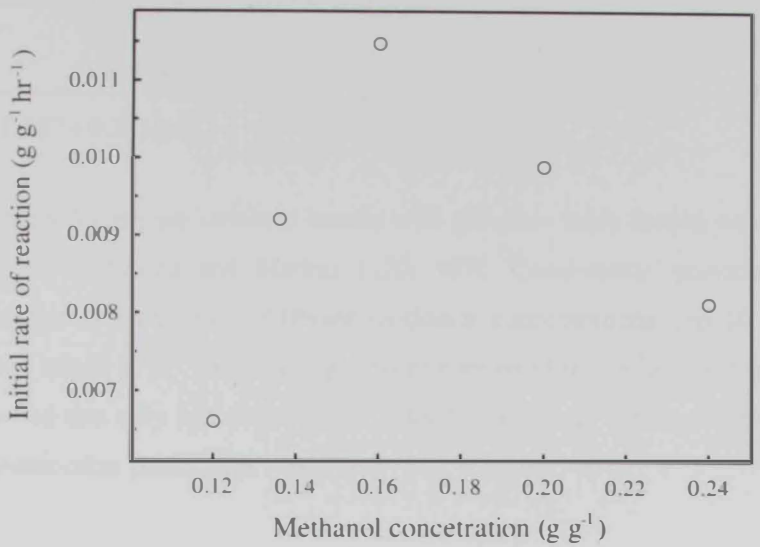

Figure 4.18: Effect of increasing methanol concentration on initial reaction rate

Many authors verified that the kinetics studies on lipase catalyzed reactions follow ping-pong Bi Bi model with competitive inhibition of an alcohol [118-121, 199]. General ping-pong reaction rate with alcohol competitive inhibition is given in Eq. (2.3). This model was based on initial rate conditions with negligible product inhibition. To find the numerical values of kinetic parameters (v_{max}, K_M, K_F and K_{IM}), Eq.(2.3) was simplified by taking the initial fat concentration as constant as shown in Eq. (4.5). Excel solver was then used to find the parameters that result in minimum objective function. The objective function compares experimental reaction rates with the predicted ones from the proposed model (Eq. (4.6)). Eq. (4.7) shows modelled equation with estimated parameters for unity fixed fat mass concentration.

$$v = \frac{v_{max}[M]}{[M] + K_M + K_M[M]\left(1 + \frac{[M]}{K_{IM}}\right)} \qquad \text{Eq. (4.5)}$$

Where v is the initial reaction rate, [M] the initial mass concentrations of methanol,; K_F and K_M the apparent Michaelis–Menten constants for fat and methanol, respectively, K_{IM} the apparent methanol inhibition constant and v_{max} the initial maximum velocity of the reaction.

$$\text{Objectivefunction} = \sum \left(v_{\text{experimental}} - v_{\text{model}}\right)^2 \qquad \text{Eq. (4.6)}$$

$$v = \frac{0.135[\text{M}]}{[\text{M}] + 1.257 + 0.301[\text{M}]\left(1 + \frac{[\text{M}]}{0.009}\right)} \pm 8.5\times10^{-4} \qquad \text{Eq. (4.7)}$$

Table 4.5 compare obtained results with previous study results carried out in SC-CO_2 medium by Varma and Madras [120, 167]. Consistency, previous researchers' kinetic parameters units using different methanol concentrations and 10 mg Novozym 435 loading, mmol $g^{-1}hr^{-1}$ and mmol g^{-1}, were converted to g $g^{-1}hr^{-1}$ and g g^{-1}. Obtained results showed that only initial maximum velocity value was close to Varma and Madras result, whereas other parameters were far.

Table 4.5: kinetic parameters comparison of for the enzymatic reaction of different triglycerides in SC-CO_2.

Kinetic parameter	[167]		This study
	Sesame oil	Mustard oil	Lamb meat fat
v_{max}	0.137	0.194	0.135
K_M	0.063	0.0743	1.257
K_F	1.848	1.805	0.301
K_{IM}	1.898	2.775	0.009
K_{IF}	32.8	23.452	neglected

CHAPTER FIVE: CONCLUSION

CHAPTER FIVE: CONCLUSIONS

SC-CO_2 extraction was experimentally proven to be feasible and competitive to chemical solvent extraction of fat from lamb meat samples. It showed comparable yield and shorter extraction time as compared to the soxhlet extraction. It was concluded that at least 63.4-87.4 % of the initial amount of fat can be extracted within selected range of conditions. GC-FID analysis showed that the extracted fats contained mainly linoleic acid, oleic acid, linolenic acid, palmitic acid and mystric acid. It was observed that the extraction yield increased with reducing sample water content and particle size under similar operating conditions. The results also showed that SC-CO_2 extraction system works sufficiently with freeze dried and ground meat.

To perform process optimization experiments, extraction temperature, extraction pressure and SC-CO_2 flow rate were chosen as extraction parameters. Results indicated that; increasing SC-CO2 decreased extraction yield and efficiency, increasing extraction pressure increased extraction yield and efficiency whereas, extraction temperature has competing effect. Temperature affects SC-CO_2 solvation power and at the same time fat volatility. After passing 200 ml of SC-CO_2, It was found that pressure effect was insignificant in comparison to other parameters effects.

Minitab 15 was employed to fit a second-order polynomial equation to the obtained experimental data. The analysis of variance (ANOVA) indicated that the second-order polynomial model was highly significant and sufficient to represent the actual relationship between the response, extraction yield, and the significant variables, temperature and flow rate, with p-value =0.000 and a satisfactory coefficient of determination (R^2=0.89).

Biodiesel enzymatic production using biocatalysts under SC-CO_2 conditions has proved to be a high potential process with a good conversion. Maximum conversion of 49.2% was obtained at 50°C, 4:1 molar ratio, 50% loading within 25 hr reaction and optimum conversion at 50°C, 4:1 molar ratio and 30% loading. Within the optimized conditions, lipase activity was reduced with the repeating use. This unpromising result suggests for further investigation.

Summarizing, this study proved that using green catalyst and green extraction and reaction techniques could be implemented.

APPENDICES

APPENDIX A: SC-CO_2 EXTRACTION OF FATS AT DIFFERENT CONDITIONS

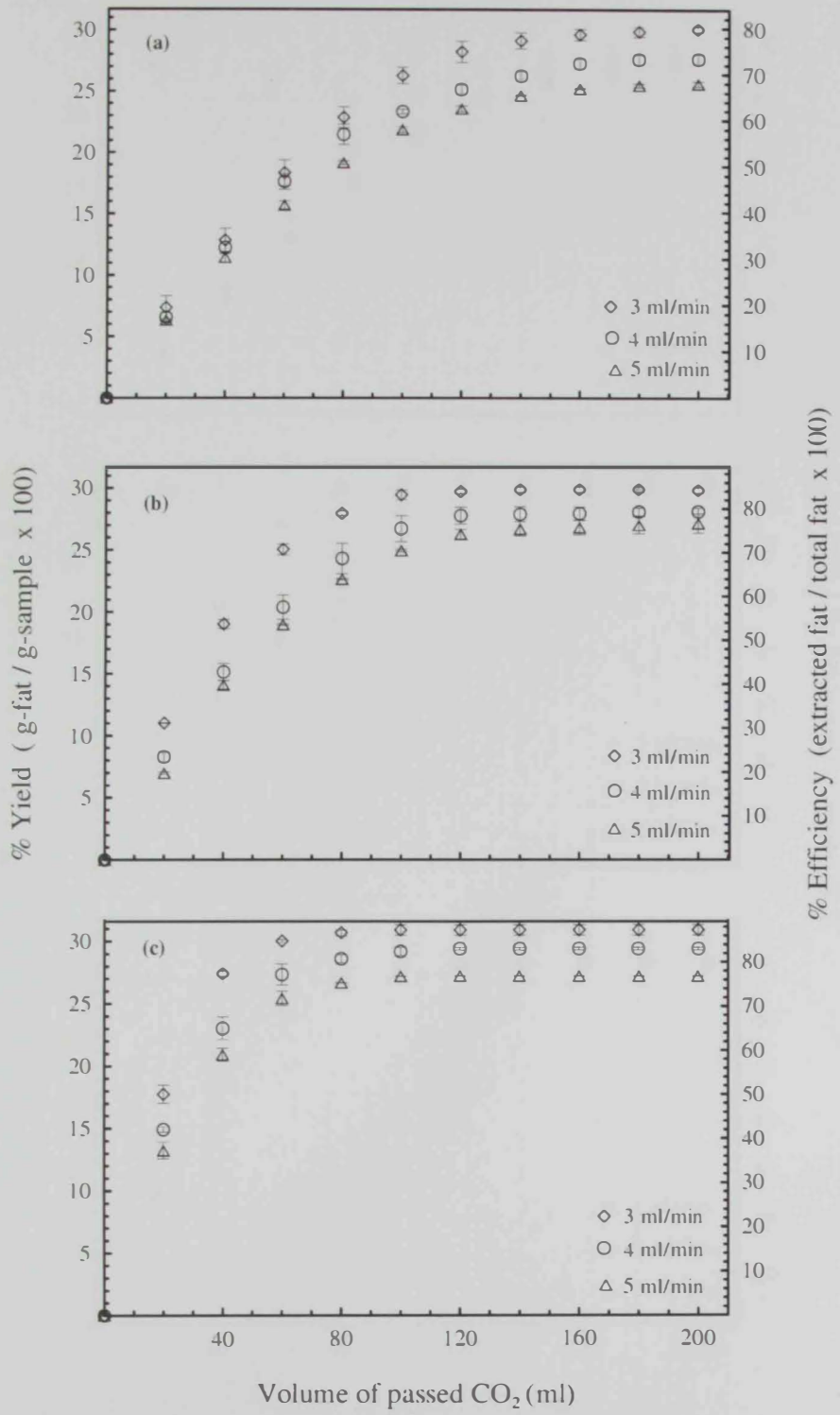

Figure A.1: Effect of SC-CO_2 flow rate at 45°C and different pressures on extraction yield and efficiency (a) 300 bar, (b) 400 bar and (c) 500 bar

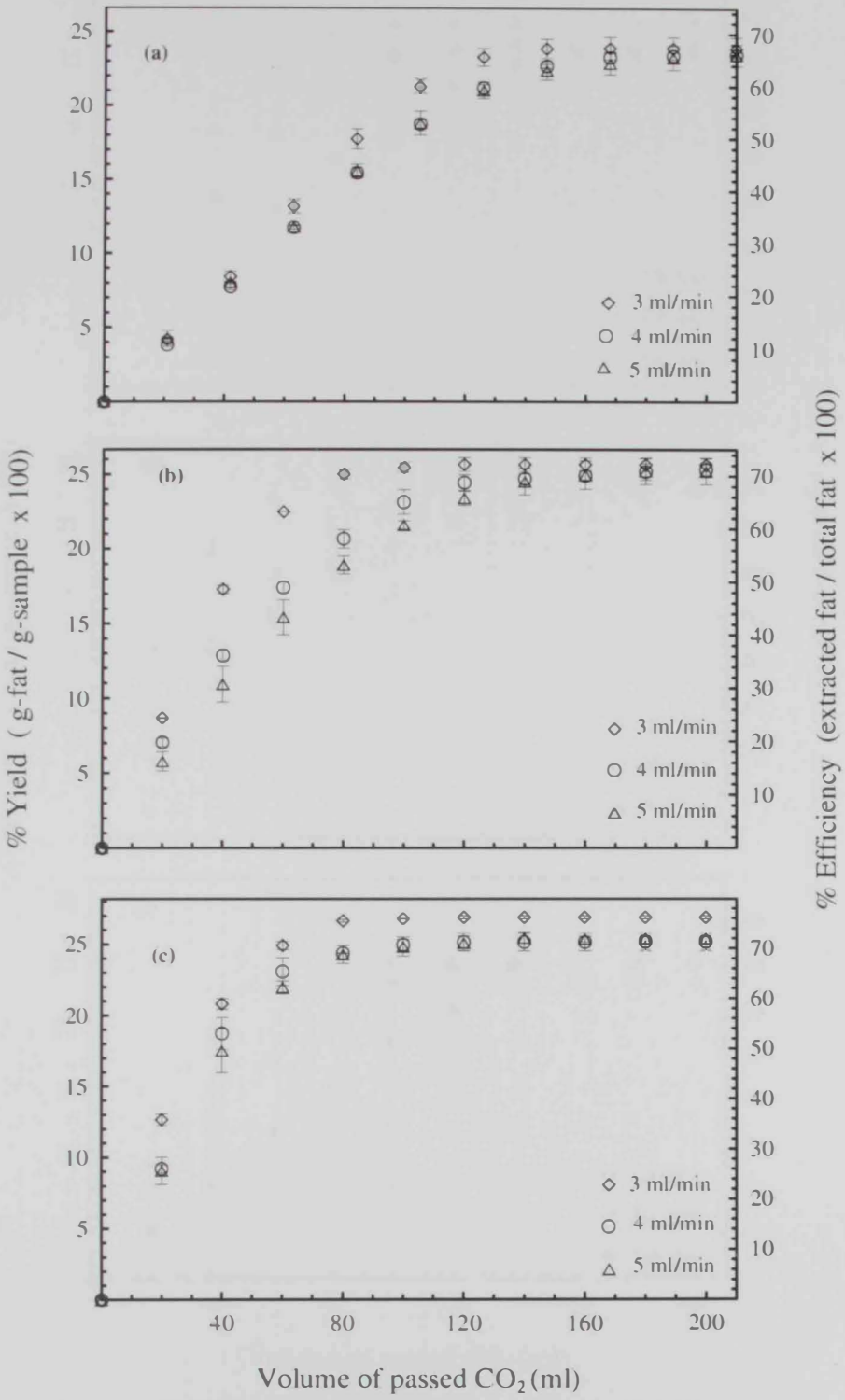

Figure A.2: Effect of SC-CO_2 flow rate at 55°C and different pressures on extraction yield and efficiency (a) 300 bar, (b) 400 bar and (c) 500 bar

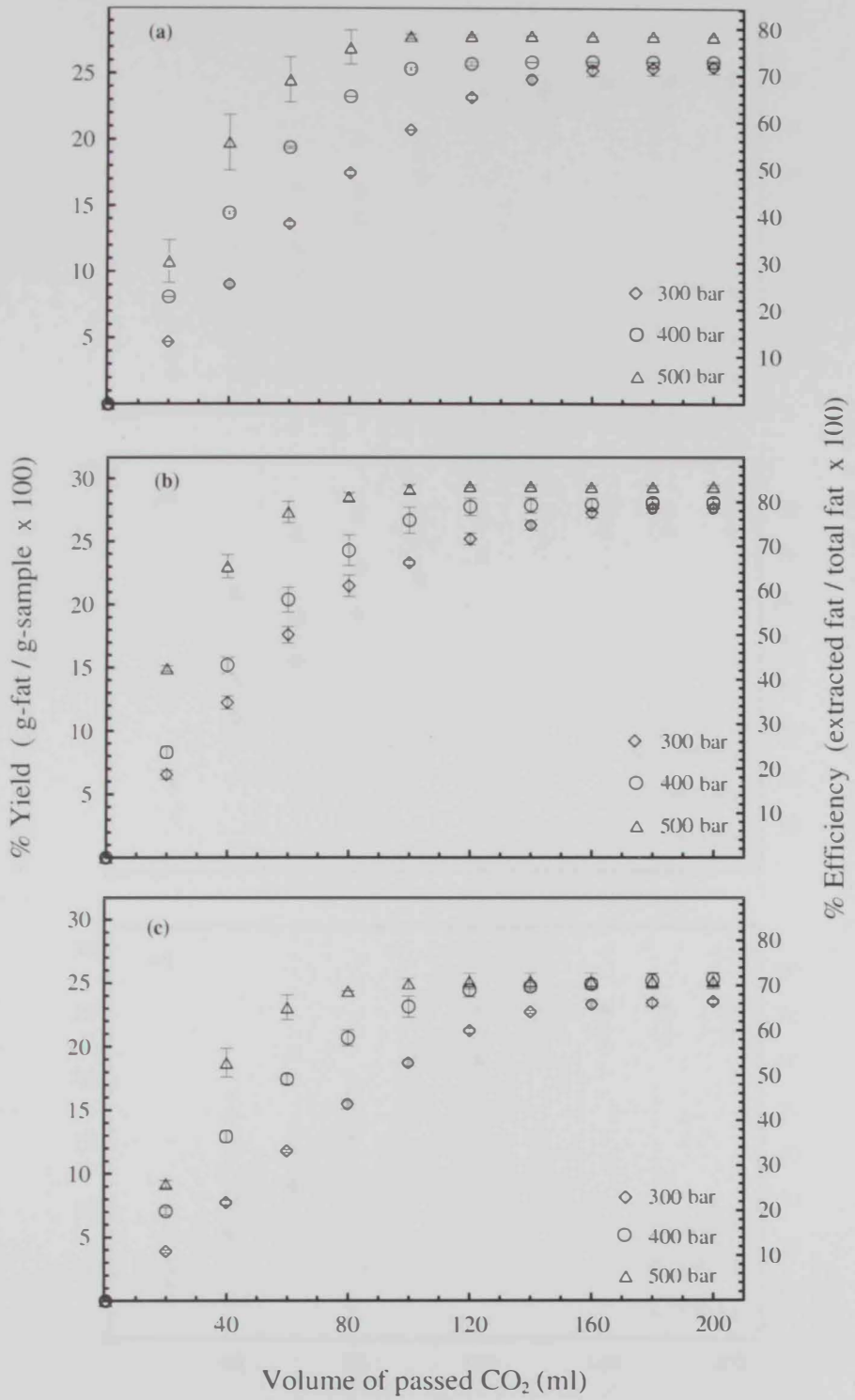

Figure A.3: Effect of SC-CO_2 pressure at 4 ml min^{-1} and different temperatures on extraction yield and efficiency (a) 35°C, (b) 45°C and (c) 55°C

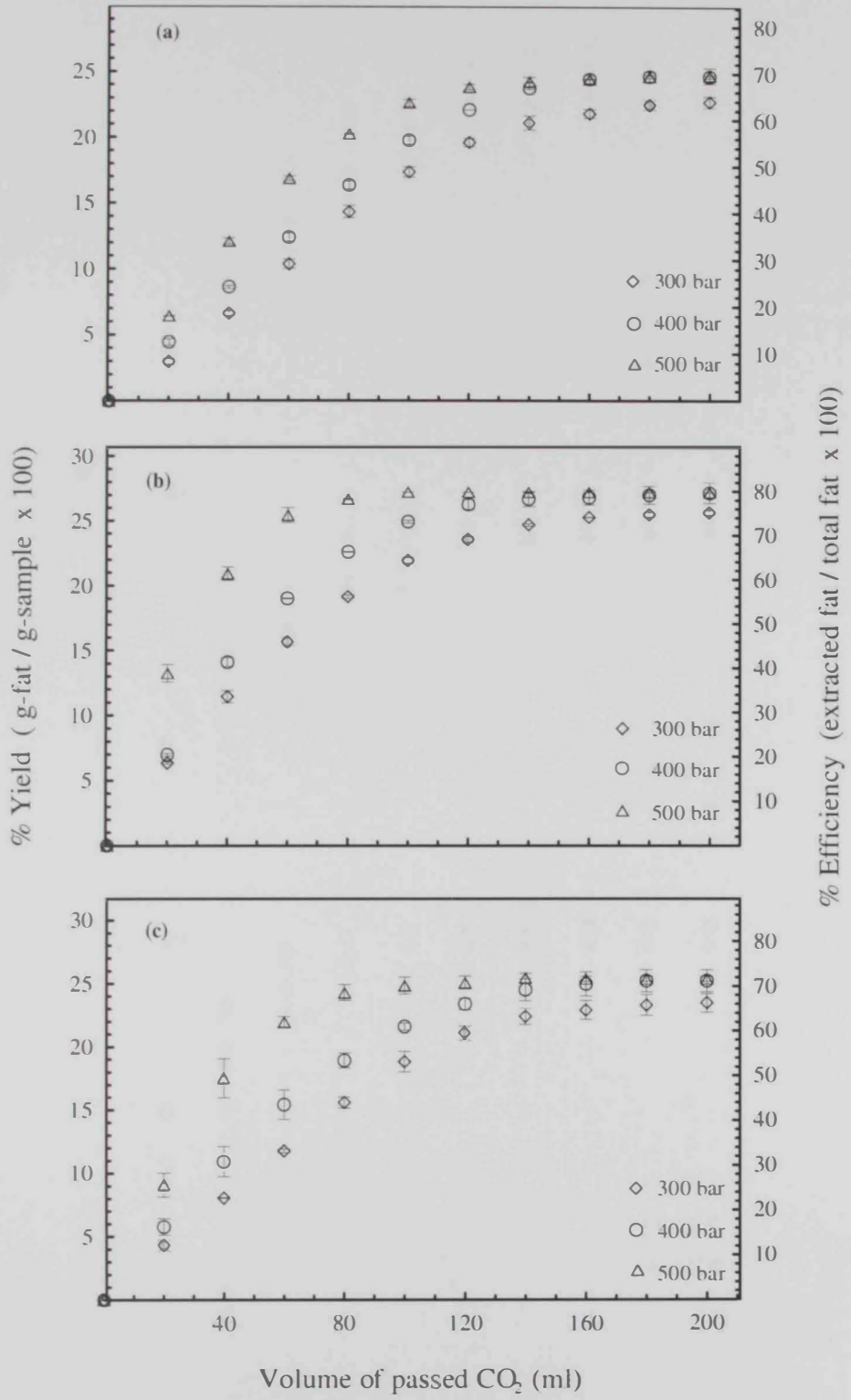

Figure A.4: Effect of SC-CO_2 pressure at 5 ml min^{-1} and different temperatures on extraction yield and efficiency (a) 35°C, (b) 45°C and (c) 55°C

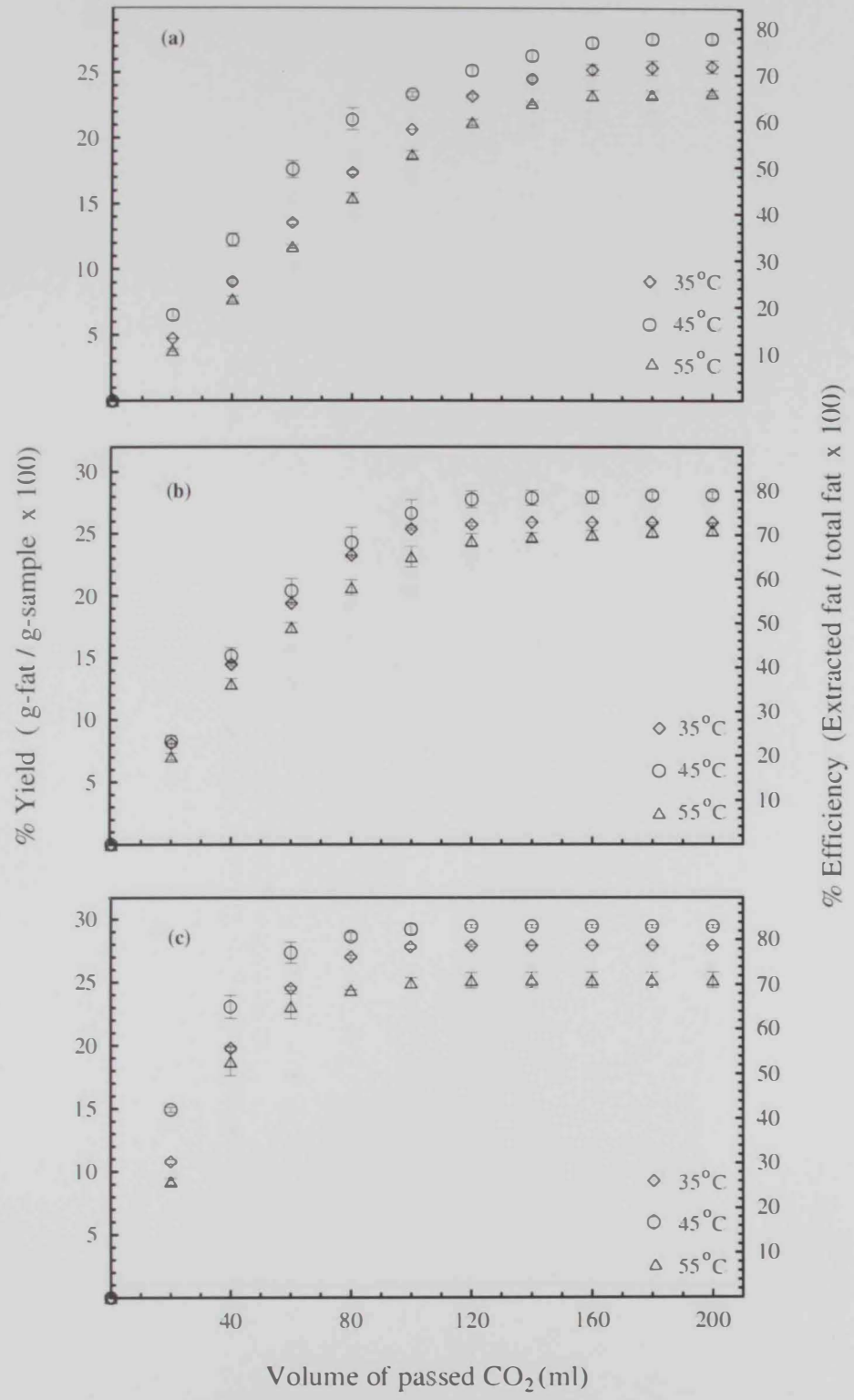

Figure A.5: Effect of SC-CO_2 temperature at 4 ml min^{-1} and different pressures on extraction yield and efficiency (a) 300 bar, (b) 400 bar and (c) 500 bar

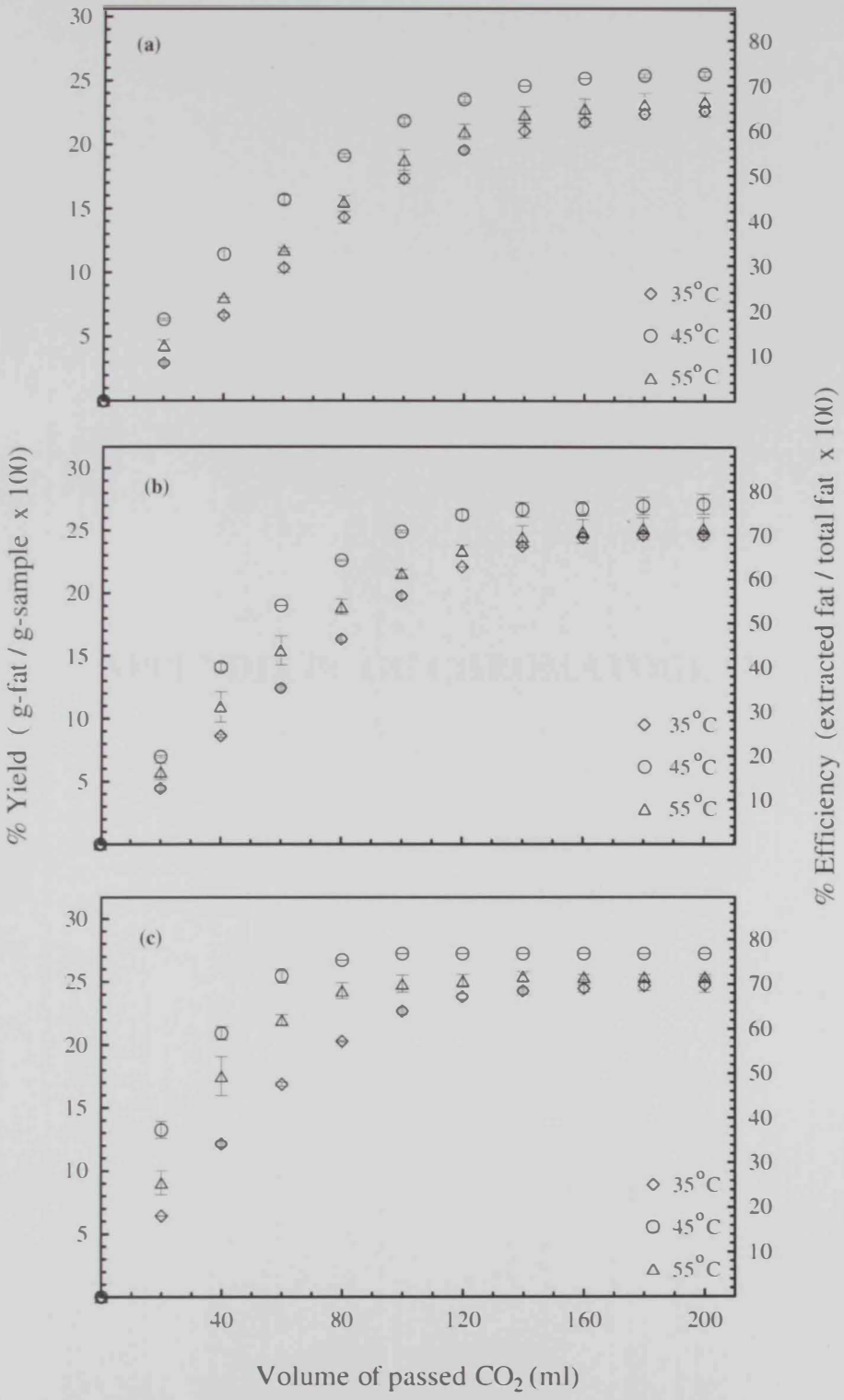

Figure A.6: Effect of SC-CO_2 temperature at 5 ml min^{-1} and different pressures on extraction yield and efficiency (a) 300 bar, (b) 400 bar and (c) 500 bar

APPENDIX B: GC CHROMATOGRAMS

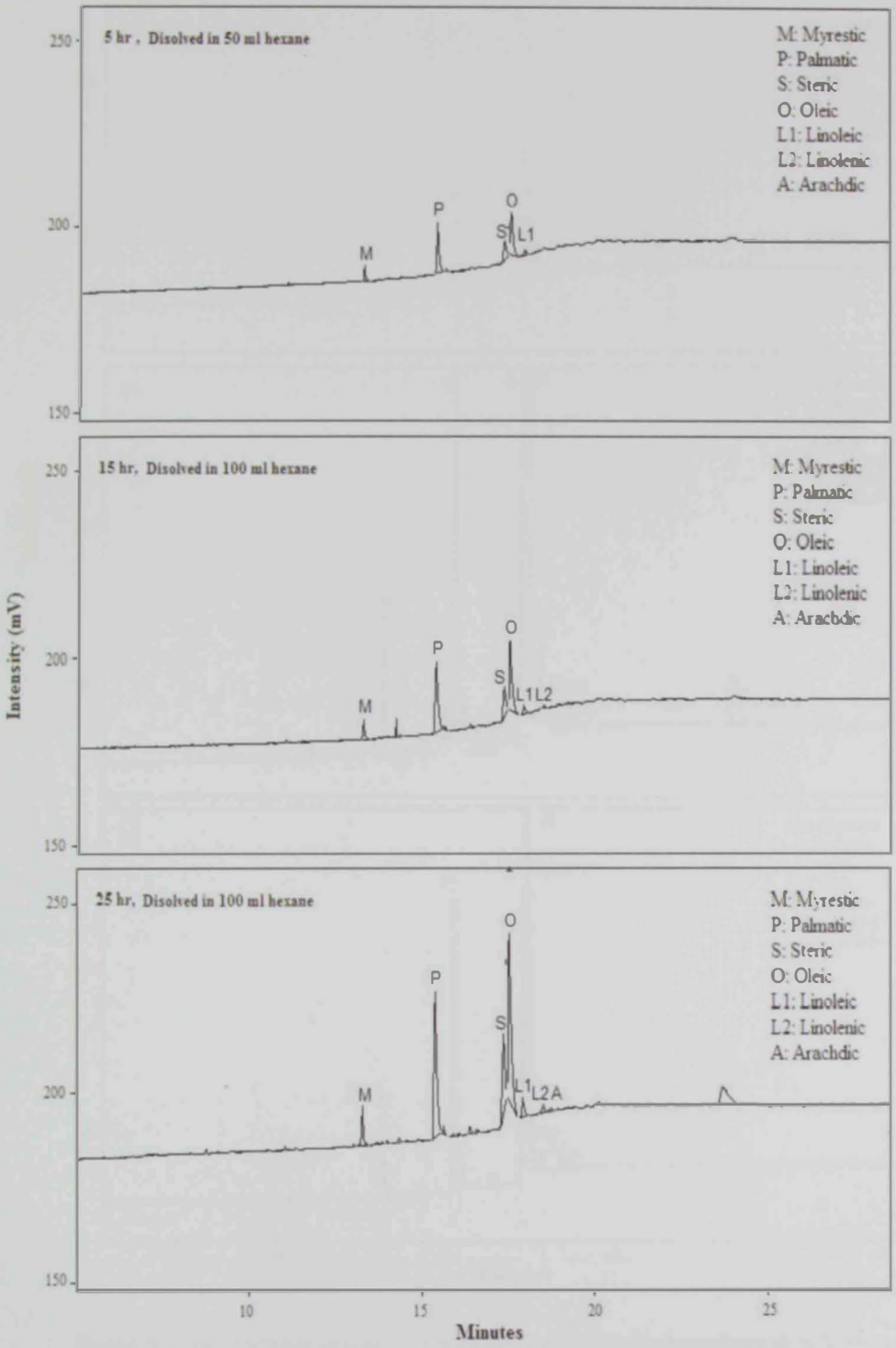

Figure B.1: GC chromatograms of transesterification reaction products at 10% enzyme loading and different reaction time

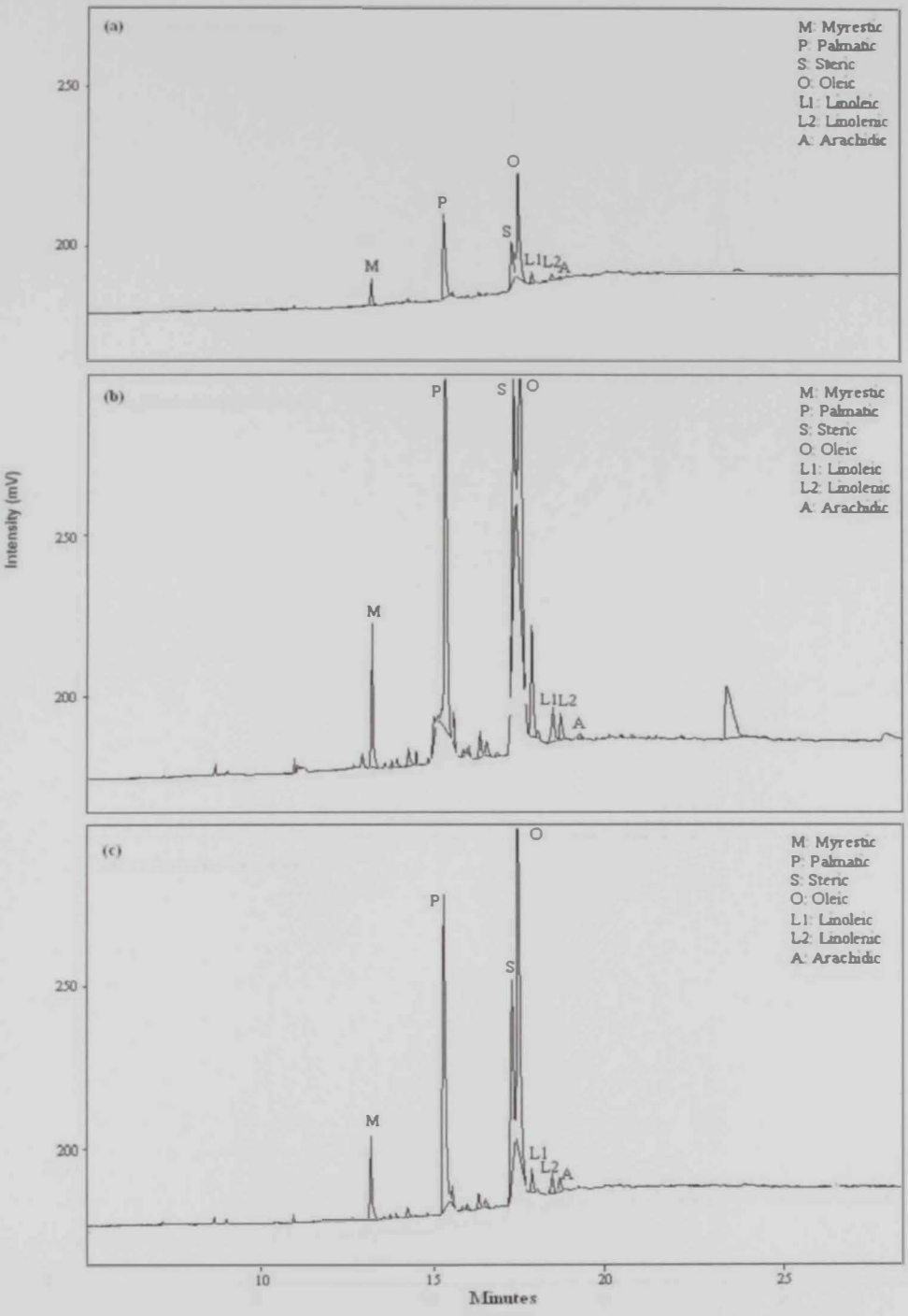

Figure B.2: GC chromatograms of transesterification reaction products at 30% enzyme loading and different reaction time

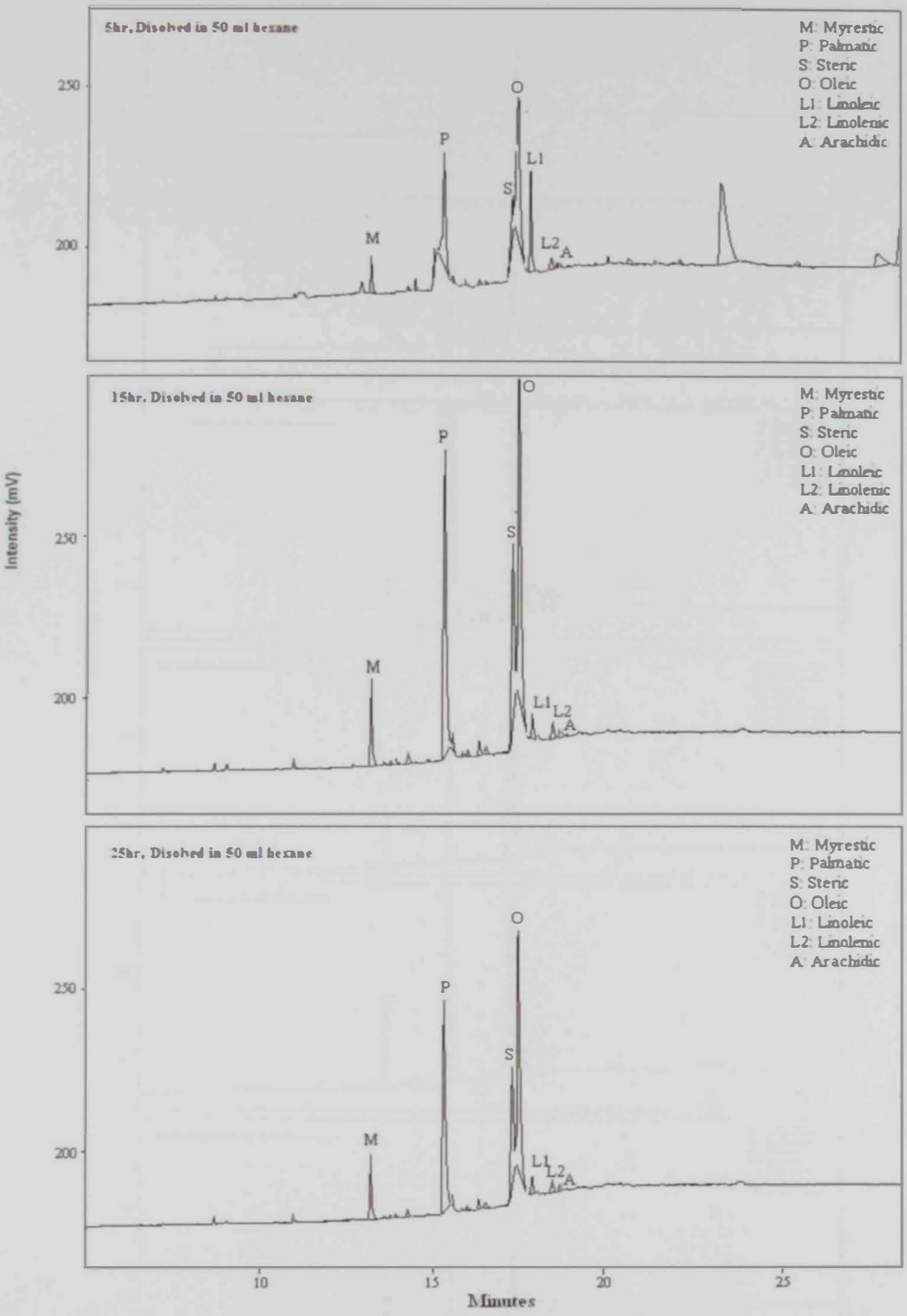

Figure B.3: GC chromatograms of transesterification reaction products at 50% enzyme loading and different reaction time

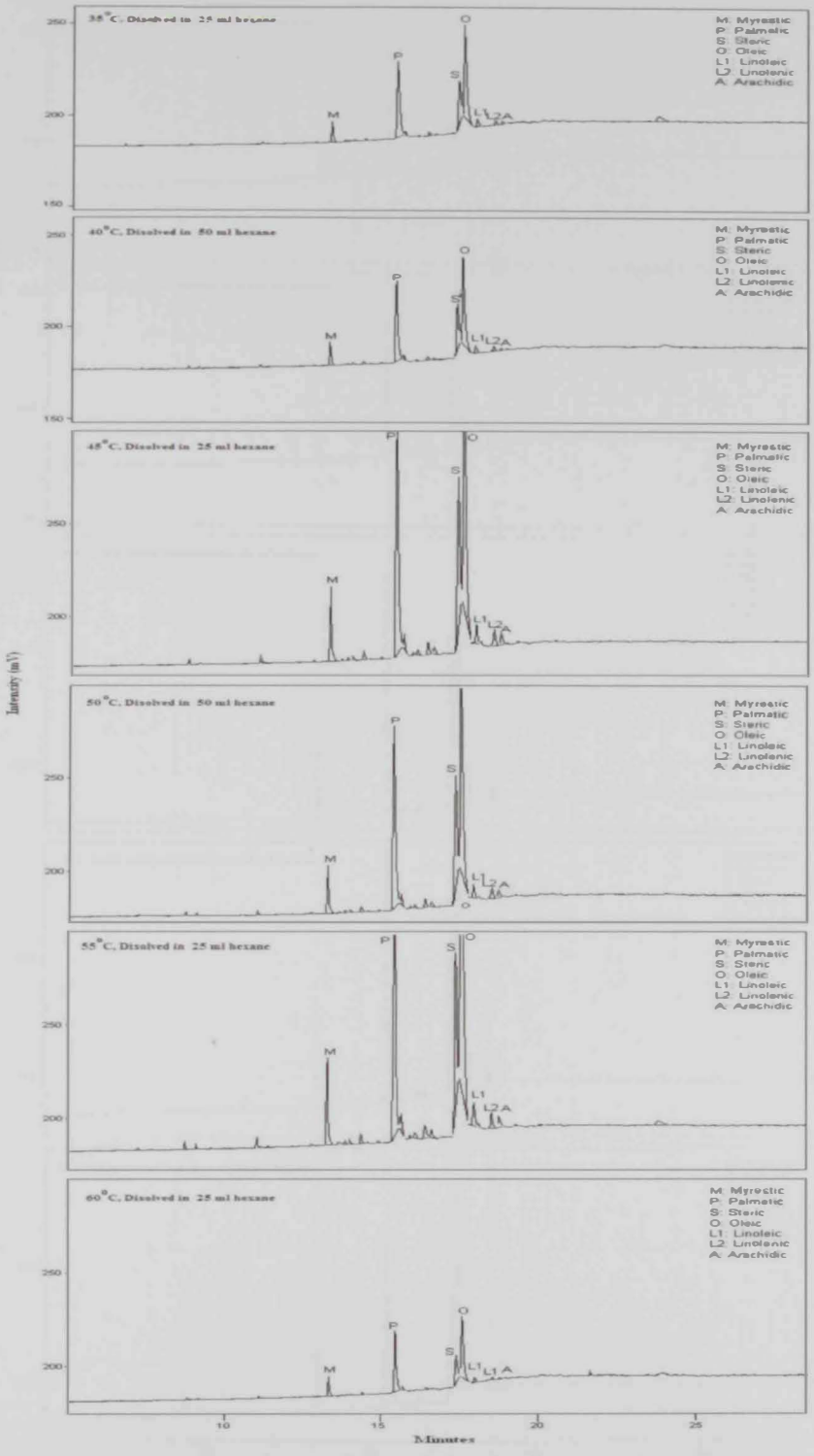

Figure B.4: GC chromatograms of transesterification products for different reaction temperatures at 4:1 molar ratio and 30% enzyme loading

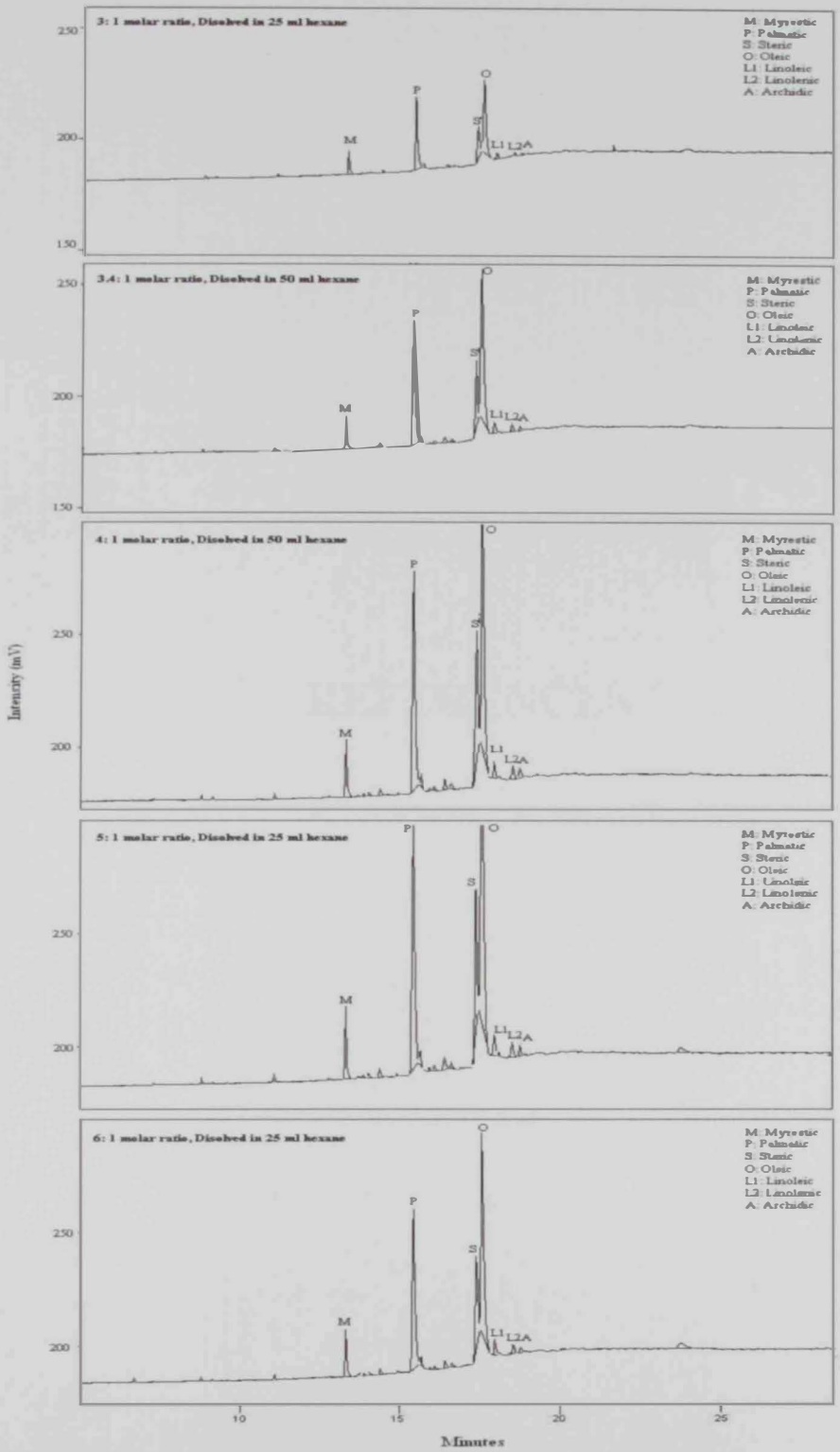

Figure B.5: GC chromatograms of transesterification products for different reaction molar ratios at 50°C and 30% enzyme loading

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ملخص الأطروحة

نظرا لإنخفاض إحتياطيات الوقود الأحفوري والتأثيرات البيئية المرتبطة بإستخدام الوقود البترولي، قُدم الوقود الحيوي أو ما يُدعى ب " الديزل الحيوي" كبديل مناسب لكونه مستديم و صديق للبيئة. في هذه الدراسة، تم التحقيق من جدوى الإنتاج الأنزيمي للديزل الحيوي من الدهون الحيوانية (دهن اللحم الحيواني) بإستخدام تقنية الموائع الفوق الحرجة (Supercritical Fluids) كمذيب و وسط للتفاعل و الليباز المثبت (Immobilized lipase) كإنزيم. علاوة على هذا، هدفت الدراسة إلى تحسين ظروف إستخلاص الدهن من اللحم و تحديد أفضل الظروف المناسبة للإستخلاص الأمثل. كذلك تحديد أمثل الظروف لإنتاج الديزل الحيوي الأمثل. يكمن الجديد في الدراسة في الإستفادة من الدهون الحيوانية الغير المرغوبة كمادة خامة للحصول لى الديزل والمحافظة على جودة اللحم المستخلص للإستخدام الآدمي.

لتحقيق أهداف الدراسة، تم استخدام دهن لحم الظأن كمصدر،ثاني أكسيد الكربون الفوق الحرج كمذيب ووسط للتفاعل، Novozym 435 كعامل حيوي حفاز، التحليل الكروموتغرافي الغازي المزود بكاشف لتأين اللهب (GC-FID) لتحديد تركيب الدهن و كمية الديزل الحيوي المُنتَج من التفاعل الأستري. لقد تمت دراسة تأثير حرارة الإستخلاص (٣٥، ٤٥ و ٥٥ °م) ، ضغط الإستخلاص (٣٠٠،٤٠٠ و ٥٠٠ بار) و معدل التدفق الحجمي لغاز ثاني اكسيد الكربون (٣، ٤ و ٥ مل لكل دقيقة). على حصيلة الدهن و كفاءة الجهاز على الإستخلاص بالمقارن مع حصيلة الدهن المستخلص باستخام المذيب العضوي (هكسان). أما تعظيم حصيلة الإستخلاص فقد دُرست بإستخدام منهجية استجابة السطح (Response Surface Methodology). تم إستخدام البرنامج التحليلي (Minitab 15) للتحليل الإحصائي للحصيلة. أما في التفاعل الأستري، لقد تمت دراسة تأثير درجة حررة التفاعل (٣٥-٦٠°م) ، النسبة المولية بين المتفاعلات (١:٣-١:٦) ، كمية الانزيم المستخدم (٠,٠٥ ، ٠,١٥ و ٠,٢٥ملجم) ، فترة التفاعل و الإستخدام المتكرر للإنزيم (٧ تكرارت) .كذلك، لقد تم دراسة حركية التفاعل عن طريق دراسة معدل سرعة التفاعل الأولي و إستخدام (Ping Pong bi bi model).

أشارت نتائج الدراسة أن إستخلاص الدهن بإستخدام ثاني اكسيد الكربون الفوق حرج يتطلب التعامل مع عينة جافة و بإستخدام درجات حرارة معتدلة بحيث أنه يمكن أن يكون ميزة إيجابية من إستخدام تقنية الفوق حرج. أثبت ثاني أكسيد الكربون قدرته على إستخلاص ٨٧،٤ % من محتوى الدهن الكلي بعينة لحم مجففة عند الظروف ٤٥°م، ٥٠٠ بار و ٣مل لكل دقيقة. أما التحليل الإحصائي أشارت أن عامل الحرارة و معدل تدفق الحجمي لثاني أكسيد الكربون لهما الدور الأكبر في إستخلاص الدهن بإمرار ٢٠٠ مل من ثاني أكسيد الكربون في حين للضغط تأثير ضئيل. علاوةً، أثبتت التفاعل الأستري في وجود ثاني أكسيد الكربون الفوق حرج كوسط تفاعلي و الليباز المثبت كعامل قدرتها على تحويل الدهن إلى الديزل الحيوي بالتفاعل مع الميثانول بمعامل تحويل يُقارب 40% تحت

الضروف المثلى : ٥٠°م، ٠.٠٥ ملجم إنزيم، ٤:١ نسبة مولية بين المتفاعلات خلال ٢٥ ساعة تفاعلية. بالمقابل أظهرت الدراسة أن الإستخدام المتكرر أفقدت Novozym 435 نشاطها.

جامعة الإمارات العربية المتحدة

عمادة الدراسات العليا

برنامج ماجستير علوم وهندسة البترول

الإنتاج الإنزيمي للديزل الحيوي من الدهون المستخرجة من لحوم الظأن بإستخدام ثاني أكسيد الكربون الفوق حرج

رسالة مقدمة من الطالبة

حنيفة عيسى طاهـــر

إلى

جامعة الإمارات العربية المتحدة

إستكمالاً لمتطلبات الحصول على درجة الماجستير في علوم وهندسة البترول (هندسة كيميائية)

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